Variation in postpartum maternal care programs the development of neuroendocrine and mesolimbic dopamine pathways in female offspring

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Abstract

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Variation in adult rat maternal behavior is predicted by the experience of maternal licking and grooming (LG) in infancy, such that females that experience high levels of LG (High LG) during postnatal development typically themselves become High LG dams, and females that experience low levels of LG become Low LG dams. Experience of high maternal LG also predicts elevated estrogen receptor-alpha (ERα) and oxytocin receptor levels in brain regions critical for maternal behavior such as the medial preoptic area of the hypothalamus. The first series of experiments within this thesis demonstrates that these neuroendocrine differences in ERα-immunoreactivity and mRNA emerge in offspring during the postnatal period (postnatal days 0-21). These studies also show postnatal emergence of epigenetic alterations of the ERα gene (*Esr1*), including DNA methylation and chromatin remodeling, in response to maternal care. Furthermore, this research reveals sensitive periods during postnatal development for maternal LG to affect gene expression and onset of maternal behavior in juvenile offspring. The mesolimbic dopamine system is also critically implicated in adult maternal behaviors, and was hypothesized to be responsive to variation in maternal LG. A second series of studies demonstrate that low or high levels of maternal LG predict levels of dopamine neurons in the

ventral tegmental area, an effect that emerges during the postnatal period and lasts through adulthood. These neurobiological changes within the ventral tegmental area may be shaped by maternal LG-associated effects on postnatal levels of transcription factors that contribute to development of the mesolimbic dopamine system. Reward-directed behaviors known to be dependent upon mesolimbic dopamine function were also found to be different among offspring of High or Low LG dams. Finally, a third series of experiments reveal that over-expressing ERα in the medial preoptic area beginning early in postnatal development is sufficient to enhance maternal behaviors, and increase the level of dopaminergic cells in the ventral tegmental area in offspring reared by Low LG dams to the level of those reared by High LG dams. This finding suggests that ERα is a mediating factor for the effect of maternal LG on offspring maternal behavior. Together, these studies show that the quality of the maternal environment early in life programs long-lasting alterations in two brain systems critical for complex behaviors such as maternal and reward-directed behaviors.

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Chapter 1: Introduction

A central question of neurobiology is how experiences can shape the brain and behavior. The brain is particularly sensitive to environmental experiences early in life while it is developing (Glaser, 2000). Among altricial mammals, maternal care is the predominant experience in early postnatal development, and as such has the potential to significantly influence offspring brain development and behaviors. The current research sought to understand the timing and mechanisms by which variation in the quality of early postnatal maternal care influences development of offspring hypothalamic neuroendocrine and mesolimbic dopamine systems, two brain systems critical for complex behaviors including maternal and reward-directed behaviors.

Maternal behavior

Variations in human parental and maternal care

There is wide variation in human parenting, ranging from abusive and neglectful parenting on one end of the spectrum to sensitive, supportive, and stimulating parenting on the other end. In 2009, approximately 1% of children aged 17 or younger were victims of some form of maltreatment, including neglect, physical abuse, sexual abuse, emotional abuse, and medical abuse (Child_Trends, 2011). Rates of child maltreatment have remained steady over the decades, despite efforts to reduce maltreatment, indicating that it is a stable behavior in the population (Leventhal et al., 1993; Child_Trends, 2011). The effects of maltreatment on children's physical and mental health are significant. Children who experience adversity often

display “problem behaviors” early in childhood (McCue Horwitz et al., 2012; Singh and Ghandour, 2012). As adults, those who experienced childhood maltreatment are more likely to have poorer physical health including: heart disease, hypertension, ulcers, pain sensitivity, dysregulated hormonal response, and increased reactivity to stress (De Bellis et al., 1994; Russek and Schwartz, 1997; Heim et al., 2008). Victims of child abuse are at significantly greater lifetime risk of at least one mood or psychiatric disorder, including: major depression, anxiety, alcoholism, drug abuse, attention-deficit/hyperactivity disorder, posttraumatic stress disorder, bipolar disorder, schizophrenia, eating disorders, personality disorders, and suicidal risk (Holmes and Robins, 1987; Riggs et al., 1990; Heim et al., 1997; Bensley et al., 1999; Heim and Nemeroff, 2001; Heim et al., 2008; Hahm et al., 2010; Heim et al., 2010; Enoch, 2011; Scott et al., 2012), and these risks increase in a “dose dependant” manner (Jonson-Reid et al., 2012). Cognitive impairments such as impaired memory recall, lower IQ, and impaired responses to rewards are also associated with childhood maltreatment (Dillon et al., 2009; Cicchetti et al., 2010; Jaffee and Maikovich-Fong, 2010). Furthermore, childhood maltreatment has been associated with differences in brain structure and physiology including decreased hippocampal and medial prefrontal cortex volumes, increased amygdala volume, and weak basal ganglia activation (Dillon et al., 2009; Mehta et al., 2009; Van Harmelen et al., 2010; Teicher et al., 2012).

Adoption away from an adverse parenting environment is beneficial for improving long-term outcomes, within limits. Favorable effects of adoption are dependent on the amount of time spent in the adverse environment (the age adopted), indicating that there are critical periods for these experiences to have an effect. Children who experienced abuse or neglect prior to adoption generally fare far better than those who remain in abusive homes (Fergusson et al., 1995;

Johnson, 2002). However, they are more likely to have long-term behavioral difficulties than non-abused children, with increased risk associated with older age at adoption (Rushton and Dance, 2006). A number of studies examined children institutionalized in Romanian orphanages and adopted by western families in the early 1990's in order to understand the effects of impoverished environments early in life (Ames, 1997; Rutter, 1998; Johnson, 2002; Iftene and Roberts, 2004). Children from the orphanages had extreme developmental delays at the time of adoption, including delays in motor, social, language, and cognitive domains (Ames, 1997; Rutter, 1998; Johnson, 2002). Even three years after adoption, previously institutionalized children were more likely to be aggressive, antisocial, rageful, and oppositional (Ames, 1997; Johnson, 2002). Interestingly, a "dose dependent" relationship between the duration of institutionalization and IQ was found in the first years after adoption, with children adopted at 4 months or younger not significantly different from age-matched control children (Rutter, 1998; Johnson, 2002). Duration of institutionalization has been associated with lower cognitive achievement and altered brain amygdala volumes even 10-16 years after adoption (Johnson, 2002; Mehta et al., 2009), underscoring the fact that early life experiences can have long-lasting impacts on the brain.

Although it is perhaps not surprising that extreme forms of early adverse experiences would induce significant changes in child development, long-lasting effects are also observed within the positive range of early experiences. Examples of favorable outcomes from early childhood enrichment are found in the Early Head Start and Head Start programs. Early Head Start is a federally funded community-based program for low-income ("at risk") families or single parents. It promotes prenatal health, healthy family functioning, and support for children up to age three. Head Start offers educational, health, nutritional, and other support for three to

five year old children and their families. Children from these programs were found to have many long-lasting cognitive and behavioral benefits including: lower levels of aggressive behaviors, higher levels of sustained attention in play, greater engagement with their parents, less negativity towards their parents, improved vocabulary and pre-writing skills, improved math skills, and improved health status (Child_Trends, 2010). These favorable outcomes may be mediated at least in part by improved parenting behaviors, including: providing greater warmth and support, spending more time playing, providing more educationally stimulating home environments, providing more language learning support, being more likely to read daily with their child, and being less likely to spank their child (Child_Trends, 2010). **Despite the diversity of known long-term effects of early childhood experience, our knowledge of the neurobiological mechanisms underlying these outcomes is still limited.**

Rats as a model system to study maternal behavior

Although studies in humans have provided insight into these issues, experimental evidence for the neurobiological and molecular impact of early life experiences has relied primarily on laboratory studies in rodents. Moreover, studies in laboratory rodents suggest that variations in parental care within the normal range can likewise shift developmental trajectories. The use of rodents allows experimental manipulation of early life experiences, access to brain tissue for region-specific analysis, and the ability to study brain and behavior of mothers (dams) and offspring across multiple generations. In rodents, as in most mammalian species, the mother is the caregiver. Dams provide offspring with basic care, feeding, cleaning, tactile stimulation, and temperature regulation, all of which are essential for infant survival. The behavioral repertoire of postpartum rat dams is complex and involves nest building, retrieving pups to the

nest, nursing, and licking/grooming (LG) pups, in addition to non-pup-directed behaviors including self-grooming, eating, and drinking (Fleming and Rosenblatt, 1974; Myers et al., 1989). Nest-building and retrieval of pups back to the nest serves to keep the pups warm and facilitates nursing of a single group so that all pups have the opportunity to suckle when the dam is on the nest (Rosenblatt and Lehrman, 1963; Croskerry et al., 1978). Nursing serves primarily to nourish offspring (Lynch, 1976) as well as regulate pup heart and respiratory rates (Hofer, 1973). During nursing, dams typically adopt a rigid arched-back posture, called kyphosis (Stern, 1996), that allows sufficient rooms for pups to move and suckle. Milk let-down is thought to occur exclusively during this arched-back kyphosis posture, as opposed to when a dam lies on top of the litter ("blanket nursing") or on her side ("pig nursing"). LG is a form of tactile stimulation that serves to clean the pups, alert pups to nursing opportunities, and facilitates pup urination, defecation, and sexual development when directed at the anogenital region (Rosenblatt and Lehrman, 1963). While nursing and LG behaviors often co-occur, previous studies have found them to vary independently. Female rats unable to nurse will still crouch and groom pups, and undernourished pups elicit increased LG from dams regardless of the dam's ability or frequency of nursing (Lynch, 1976). Likewise, dams with reduced oral feedback (by tongue anesthesia) groomed pups less but were faster to crouch over young pups to nurse, and pups were sufficiently stimulated by non-licking and ventral skin contact to nurse (Hofer et al., 1976; Stern and Johnson, 1989), demonstrating both the importance of tactile contact prior to nursing and the ability to alter each independently.

LG behavior can be quantified with extensive home-cage observations, revealing a natural distribution in the amount of time engaged in pup LG (Champagne et al., 2003a). Dams can be classified as High or Low LG based on LG frequencies of the cohort: dams with an LG

frequency one standard deviation (SD) or more above the mean of the cohort are “High LG” and dams with and LG frequency one SD below the mean are “Low LG.” Importantly, contact with pups is similar among Low and High LG dams, and there are no differences in litter size, gender composition, or pup growth (Champagne et al., 2003a).

Variation in postpartum maternal LG has implications for the developing offspring brain. Offspring reared by a Low LG dam are found to exhibit heightened sensitivity to stressors in adulthood (Caldji et al., 1998; Francis et al., 1999). Interestingly, variation in LG within and across litters may have opposing effects on offspring anxiety-like behavior. Offspring from litters receiving more LG were more exploratory in novel environments than those from litters that received less LG, but within litters the effect of the level of LG on anxiety-like behavior was reversed (Cavigelli et al., 2010), indicating that while there is a main effect of litter/dam LG, further variation in offspring behavior may be a result of within-litter LG variation. Activation of the hypothalamic-pituitary-adrenal (HPA) axis stimulates physiological and behavioral responses to stressors, while glucocorticoid receptors (GR) in the hippocampus feed back to the HPA axis to reduce activity and stress response (Meaney et al., 1991). Offspring reared by Low LG dams are observed to have reduced levels of hippocampal GR in infancy, which persists into adulthood, and is associated with elevated adult HPA activity (Francis et al., 1999). These neurobiological and physiological effects may account for the behavioral inhibition that is observed among offspring of Low LG dams and may have implications for long-term health outcomes. These behavioral outcomes are likely to emerge developmentally (although there have yet to be developmental studies of stress/behavioral reactivity using this model) coincident with the maternally induced changes in neurobiological targets associated with the HPA response to stress.

The experience of Low LG compared to High LG has broad effects on the brain and behavior of offspring that extend beyond the changes in stress responsivity. Low LG is associated with impaired learning/memory, reduced hippocampal plasticity, and changes in gene activity within the hippocampal region (Liu et al., 1997; Caldji et al., 1998; Liu et al., 2000a; Bagot et al., 2009). Among adult female offspring, variation in the experience of LG is associated with variation in neuroendocrine systems and maternal/reproductive behaviors (Champagne et al., 2001; Champagne et al., 2003b; Champagne et al., 2006; Champagne and Meaney, 2007; Cameron et al., 2008a). Moreover, cross-fostering (adoption) at birth indicates that it is the frequency of maternal LG experienced during postnatal development, rather than prenatal or genetic factors, that is critically related to these maternally-induced effects seen in adulthood (Francis et al., 1999; Champagne et al., 2006). Overall, it is evident that variation within the normal range of parent–offspring interactions can have profound effects on numerous neurobiological and neuroendocrine circuits leading to persistent changes in behavior. The challenge of this research is in understanding how such long-term effects can be achieved.

Transmission of maternal behavior across generations

LG behavior of Long Evans rat dams is stable across multiple litters and predicts daughter and granddaughter LG behavior, suggesting that these developmental effects persist across generations (Champagne et al., 2003a). Thus, in stable environmental conditions, females reared by a Low LG dam are more likely to engage in low levels of LG with their own offspring, and females reared by a High LG dam are more likely to be High LG mothers themselves. Female pups cross-fostered from High to Low or Low to High LG dams display the LG phenotype of their foster mother rather than biological mother, indicating that this behavior is

transmitted across generations behaviorally, rather than by genetic or *in utero* effects. [It should be noted, however, that social experiences beyond the neonatal period can also shift the development of maternal behavior as the offspring of high LG dams can become Low LG when housed in social isolation and the offspring of low LG dams can become High LG when housed in social enrichment (Champagne and Meaney, 2007).] Levels of hormone receptors in the hypothalamus associated with adult maternal behaviors are also predicted in female offspring by the quality of maternal care received in infancy (Francis et al., 2000; Champagne et al., 2001; Francis et al., 2002; Champagne et al., 2006), suggesting a mechanism by which the experience of care may be transmitted behaviorally across generations.

The critical role of LG as opposed to other aspects of rodent maternal behavior in shaping offspring outcomes is suggested by artificial rearing studies and manipulations of the tactile interactions between dams and offspring. Fleming and colleagues have investigated the causal role of tactile stimulation in shaping offspring brain and behavior by rearing pups from PN4 until weaning age in isolation. Artificially reared pups were fed through a stomach tube at similar intervals as nursed pups in a temperature controlled environment to minimize differences in those critical aspects of the maternal environment, and thus only tactile stimulation was manipulated. Artificially reared pups were either minimally stimulated or received body and anogenital tactile stimulation by paintbrush stroking five times per day. As mothers, artificially reared dams retrieved fewer pups and engaged in less pup LG compared to standard-reared dams, which was partially reversed by supplemental tactile stimulation (Gonzalez et al., 2001; Gonzalez and Fleming, 2002). These behavioral deficits were accompanied by reduced activation of brain regions implicated in maternal care (Gonzalez and Fleming, 2002). Daughters of artificially reared dams also displayed deficits in maternal behavior, indicating a

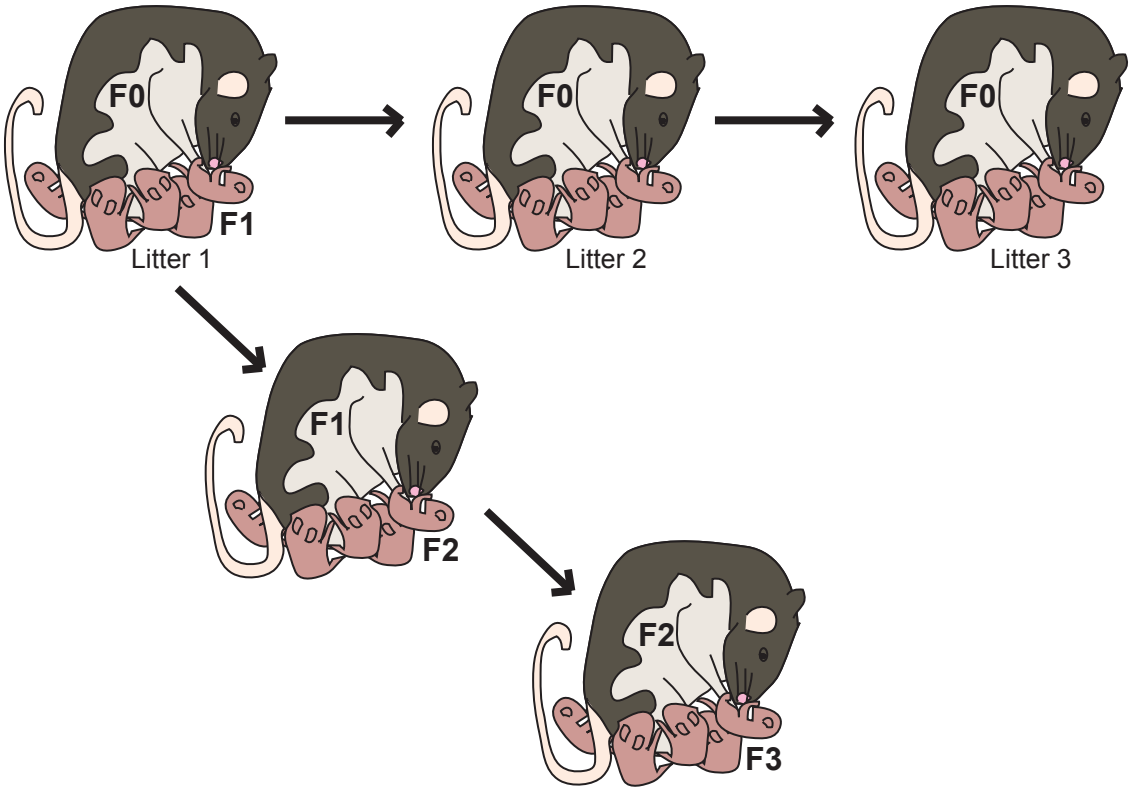
cross-generational effect of early tactile stimulation (Gonzalez et al., 2001). Taken together, these findings suggest that early life tactile stimulation in the form of LG is a critical factor in mediating variation in maternal behavior, and the underlying neural substrates, across generations.

Stability of maternal LG across generations may be a common occurrence across species. Similar transgenerational effects on maternal LG have been observed in response to communal rearing in mice. In naturalistic settings, rodents frequently engage in communal care of offspring, where newborn pups from multiple litters are grouped together and nurtured by multiple caregivers. In the laboratory, communally nesting dams displayed increased frequencies of nursing and LG compared to standard non-communally housed dams. Female offspring that experienced communal rearing in infancy exhibit increased maternal care in adulthood, as do the female offspring of these communally reared females (Curley, 2009). Additionally, similar findings have been observed in humans and non-human primates, such that attachment experience or abuse/neglect is a significant predictor of similar parenting behaviors in the next generation (Seay et al., 1964; Harlow, 1965; Arling and Harlow, 1967; Harlow and Suomi, 1971; Fairbanks, 1989; Benoit and Parker, 1994; Maxfield and Widom, 1996; Maestripieri et al., 1999; Newcomb and Locke, 2001; Bifulco et al., 2002; Kretchmar and Jacobvitz, 2002; Maestripieri et al., 2007; Shah et al., 2010; Berlin et al., 2011; Valentino et al., 2012). An understanding of the mechanisms that mediate the transmission of variation in maternal care requires knowledge of the neural circuits that mediate maternal behavior. **It is therefore important to understand how the experience of maternal care may shape development of brain systems implicated in the expression of maternal behaviors.**

Figure 1.1 Maternal LG is stable across multiple litters and can be transmitted behaviorally across generations

The first generation of females is the F0 generation, followed by the F1, F2, F3, etc generations. A dam's frequency of LG towards her first litter is predictive of her frequency of LG towards her second and third litters. The maternal LG frequency experienced by a female in the neonatal period is also predictive of the level of maternal LG she will display toward her own litter (Champagne et al., 2003a).

Figure 1.1



The maternal brain

Pregnancy and parturition are accompanied by dramatic physical and hormonal changes that enable females to carry offspring *in utero*, give birth, provide nutrients to offspring, lactate, and express a full range of maternal behaviors. Virgin female rats do not express maternal behaviors and are often neophobic, aggressive, or cannibalistic towards pups unless hormonally primed or exposed to pups repeatedly over several days (Moltz et al., 1970; Fleming, 1986). Research on the "maternal brain" has provided significant insight into specific brain regions and neurotransmitters that are critical to the expression of maternal behavior. Two key brain systems that contribute to maternal behavior, and which may also be developmentally responsive to maternal care, will be described below: the hypothalamic neuroendocrine system and mesolimbic dopamine system.

Hypothalamic neuroendocrine pathways

Hypothalamic circuitry

Early lesion and c-fos studies identified the olfactory bulb, medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), and medial preoptic area (MPOA) (see **Figure 1.2**) as regions critically involved in active maternal behaviors (including retrieval, nest-building, and LG) (Numan et al., 1977; Fleming et al., 1983; Miceli et al., 1983; Fleming et al., 1992; Fleming et al., 1994; Fleming and Walsh, 1994; Numan, 1996; Numan and Numan, 1997; Lonstein et al., 2000). Olfactory input is critical for maternal behavior in mice, but loss of olfactory input is less disruptive in postpartum rats, indicating perhaps a role in onset but not maintenance of maternal behaviors (Lonstein and Morrell, 2006).

The olfactory bulb sends projections to the MeA, which in turn sends projections to the BNST and MPOA (Simerly and Swanson, 1986). Stimulation of the MeA delays onset of maternal behaviors, while lesioning this region facilitates maternal behavior by decreasing avoidance of pups (Fleming and Walsh, 1994; Morgan et al., 1999; Lonstein and Morrell, 2006). The BNST is one relay between the amygdala and hypothalamus, including the MPOA. Neurotoxic lesions of the BNST disrupt active maternal care behaviors including nest building and retrieval (Numan, 1996). Lesioning the PVN, which is surrounded by the BNST and MPOA in the hypothalamus, induced dramatic effects in some cases, such as cannibalism of pups at birth and disrupted nest building and retrieval (Insel and Harbaugh, 1989). The effects of destruction of the PVN on maternal behavior may be due to its high concentration of oxytocin and vasopressin synthesizing cells (Lonstein and Morrell, 2006).

Figure 1.2

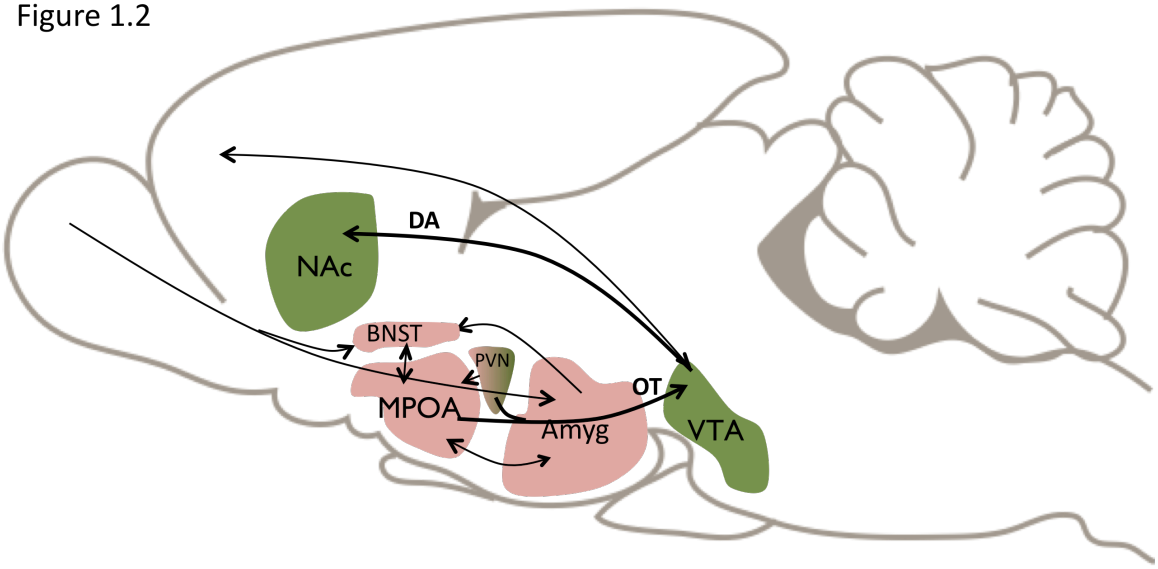


Figure 1.2 The maternal rat brain

Key rat brain regions implicated in maternal behavior and prominent projections. Oxytocin (OT) projections from the medial preoptic area (MPOA) to the ventral tegmental area (VTA), and dopamine (DA) projections from the VTA to nucleus accumbens (NAc) are highlighted. Other brain regions are the bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), and amygdala (Amyg).

Medial preoptic area

The MPOA of the hypothalamus is one of the most well-studied brain regions necessary for maternal behavior (Kalinichev et al., 2000b; Lonstein, 2003; Numan and Insel, 2003; Febo, 2005; Numan, 2006). Destruction of the MPOA in both pre- and post-partum dams, as well as in pup-sensitized virgin females, severely disrupts active maternal behaviors including nest building and retrieval (Olazábal et al., 2002; Numan and Insel, 2003). The MPOA projects to subthalamic and midbrain regions involved in locomotor behavior (Swanson et al., 1987), and thus the MPOA is believed to integrate sensory and hormonal information before stimulating brain regions necessary for the motor performance of maternal behaviors (Numan and Sheehan, 1997; Lonstein and Morrell, 2006).

Several methods have been employed to show MPOA activation in response to mother-pup interactions. *c-fos* is an immediate early gene commonly used as a marker of neurons activated in response to a specific stimulus. Increased c-fos immunoreactivity is found in the MPOA of postpartum females interacting with pups for the first time, dams reunited with pups after a long separation, and in virgin pup-sensitized females (Fleming et al., 1994; Numan and Numan, 1994). Further investigation into different components of mother-pup interaction found similar levels of MPOA activation after any tactile interaction with pups, regardless of whether nursing occurred (Lonstein et al., 1998). c-fos activation in the MPOA was found to be 2-3 times greater than in other brain regions, including the BNST, PVN, and lateral septum (LS) (Lonstein et al., 1998). Positive BOLD signal has also been identified in the MPOA (in addition to several other brain regions) of lactating rat dams stimulated by pups during fMRI recording (Febo, 2005).

The release of neurotransmitters and expression of their target receptors shift with the

hormonal priming of pregnancy and are necessary for the onset and maintenance of maternal behaviors. In particular, estrogen increases and progesterone decreases at the end of pregnancy, stimulating the production of oxytocin and its receptor to facilitate uterine contractions for birth and lactation (Moltz et al., 1970). These ovarian hormones and their receptors, oxytocin receptor (OTR) and estrogen receptors (ER, type α and type β) act within the brain to facilitate onset and maintenance of maternal behaviors (Pedersen et al., 1982; Fahrbach et al., 1984).

Oxytocin receptors and maternal behavior

Oxytocin is a small, highly conserved hormone important for many aspects of social and reproductive behaviors. Within the brain, the neuropeptide is produced primarily in the magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus, and to a lesser degree by parvocellular neurons of the PVN (Gimpl and Fahrenholz, 2001). Oxytocin transcription is highly regulated by steroids. Transcription of oxytocin is strongly stimulated by ligand-activated estrogen receptors (ERα and ERß), and also by thyroid hormone receptor-α, and retinoid acid receptors (Gimpl and Fahrenholz, 2001). Within the oxytocin gene promoter is a "composite" steroid hormone response element similar in sequence to the canonical estrogen response element (ERE), and thus estradiol is able to stimulate oxytocin transcription (Gimpl and Fahrenholz, 2001).

Oxytocin neurons project widely within the brain, and OTRs have been reported within the rodent MPOA, BNST, central amygdala, ventral tegmental area (VTA), cortical areas, olfactory system, basal ganglia, and thalamus (Gimpl and Fahrenholz, 2001). OTRs are class I $G_{q/11}$ G protein-coupled receptors (GPCRs), known to activate second messenger systems for phospholipase C-β and protein kinase C (PKC) to increase intracellular calcium release and

affect many cellular responses including neurotransmitter release. Like the gene for oxytocin itself, the rat OTR gene also contains a palindromic ERE site within the promoter (Bale and Dorsa, 1995, 1997; Gimpl and Fahrenholz, 2001). Treatment with estrogens increases OTR levels in a dose-dependent manner in culture (Bale and Dorsa, 1997) and within the brain (Fahrbach et al., 1985; Bale and Dorsa, 1995; Pedersen, 1997).

Oxytocin receptors are significantly increased at parturition in the MPOA, BNST, and MeA (Meddle et al., 2007). Intracerebroventriclar (ICV) oxytocin administration stimulates the onset of maternal behavior in estrogen-primed ovariectomized virgin rats (Pedersen and Prange, 1979; Pedersen et al., 1982). Conversely, oxytocin antagonists infused specifically in the MPOA disrupt maternal behaviors (Pedersen et al., 1994), and peripheral oxytocin antagonists block a pup-induced increase in MPOA activity measured by fMRI (Febo, 2005). Furthermore, High compared to Low LG dams have increased OTR binding in brain regions critical for maternal care (MPOA, BNST, LS, amygdala), particularly during lactation (Francis et al., 2000; Champagne et al., 2001). OTR antagonists infused ICV eliminate LG group differences, suggesting that differences in OTR expression are functionally related to active maternal behaviors (Champagne et al., 2001).

Estrogen receptors and maternal behavior

The function and regulation of the oxytocin system is strongly dependent upon ligand-activated estrogen receptors. There are two main estrogen receptor isoforms, ERα and ERß. Estrogens (estradiol/ E2, estrone, estriol) are gonadal steroid hormones. Estradiol is the most abundant and potent estrogen and is synthesized from testosterone by aromatase. The canonical mechanism of ER activation involves estrogen binding ERs in the cell nucleus, which then

dimerize, bind EREs in target gene promoters, and thereby activate gene transcription (see Bjornstrom, 2005). ERs are able to bind ERE half-sites, and ERα, specifically, is able to bind the DNA response element for orphan receptor SF-1 (Bjornstrom, 2005). ERs also affect gene transcription via interactions with other transcription factors, such as activator protein (AP-1), and thus the number of genes transcriptionally regulated by ERs extend beyond those with promoter EREs. Estrogen has also been found to induce rapid cellular alterations attributed to membrane-associated ERs acting with GPCRs and metabatropic glutamate receptors (Bjornstrom, 2005; Dominguez and Micevych, 2010).

Transcription of ERα (*Esr1*) and ERß (*Esr2*) is stimulated through the Jak2-Stat5 (janus kinase, and signal transducer and activator of transcription) pathway. Both *Esr1* and *Esr2* have response elements for Stat5b, but only *Esr1* has a response element for Stat5a (Frasor and Gibori, 2003). ERs are regulated hormonally by prolactin, which acts via the Jak2-Stat5 pathway (Frasor and Gibori, 2003), as well as by epigenetic mechanisms discussed below. ERs are also regulated by negative feedback, such that ERα protein and *Esr1* mRNA are eventually down-regulated by estradiol activation (via the ubiquitin-proteasome pathway, and via binding with a Sin3A repressor at the proximal promoter, respectively; Alarid et al., 1999; Nawaz et al., 1999; Ellison-Zelski et al., 2009).

Although there is overlap in localization and function of the two ERs, ERα and ERß can be selectively activated, are found in distinct brain regions, and are associated with different molecular and behavioral phenotypes (Fan et al., 2010). When ERα and ERß colocalize, ERß has been demonstrated to inhibit ERα-induced effects on transcription (Lindberg, 2003). ERα expression is higher in brain regions associated with social, reproductive, and maternal behaviors, including the MPOA, BNST, periventricular nucleus, ventral medial hypothalamus

(VMH), and arcuate (Arc). ERß is the dominant receptor in the cortex, hippocampus, cerebellum, dorsal raphe, nucleus accumbens shell, ventral tegmental area, substantia nigra, and brainstem regions, and has been associated with cognition, memory, stress, motivation, and movement (Shughrue et al., 1997; Fan et al., 2010; Österlund, 2010).

The role of ERs in maternal behavior is well characterized. Systemic estradiol is able to prime maternal behaviors in virgin and pregnancy-terminated ovariectomized females (Rosenblatt et al., 1998) and even in males (Sturgis and Bridges, 1997). Estradiol implants in the MPOA facilitate virgin maternal sensitization to pups, indicating the importance of estrogen actions in this brain region (Fahrbach and Pfaff, 1986). Activated ER binding to DNA increases throughout pregnancy and remains elevated during lactation, particularly in the MPOA, concurrent with increased responsiveness towards pups (Rosenblatt et al., 1994; Rosenblatt et al., 1998). Accordingly, ERα-expressing neurons are activated (cfos-immunoreactive) in the maternal brain after pup stimulation (Lonstein et al., 2000). In biparental California mice, aromatase is elevated in the MPOA of fathers compared to virgin male mice, indicating increased estrogen activity acts within the MPOA to promote parental behavior in both sexes (Trainor et al., 2003). In addition, the *level* of ERα in the MPOA is associated with the *level* of maternal LG: higher levels of ERα mRNA are found in the MPOA of High LG compared to Low LG lactating dams (Champagne et al., 2003b). Genetic tools have also been used to assess the importance of ERα to maternal care. Mice completely lacking functional ERα display significantly reduced sexual and maternal behaviors, including rejection of mating attempts, decreased pup retrieval, and increased infanticide (Ogawa et al., 1998). In studies in which ERα was knocked down specifically within the MPOA by shRNA (Spiteri et al., 2012) or RNAi (Ribeiro et al., 2012), mice displayed deficits in sexual behavior and sexual motivation (Ribeiro

et al., 2012; Spiteri et al., 2012) and nearly abolished maternal behaviors, including pup retrieval, LG, and nursing (Ribeiro et al., 2012). Although ERs are found in many brain regions, together these studies highlight the role of ligand-activated ERα particularly within the MPOA for maternal behaviors. Though these studies highlight the importance of ERα in the MPOA for maternal behavior in general, it was not known whether targeted manipulation of this system can alter individual differences in maternal behavior and whether maternal behavior could be enhanced through manipulations of ERα in the MPOA.

Development of neuroendocrine signaling and importance for offspring

OTR is detectable in the developing rodent embryo at embryoinic day (E) 13, and within the hypothalamus at birth, but not strongly until PN3-7 (Yoshimura et al., 1996). The third postnatal week and puberty mark a critical period wherein OTR expression significantly increases in the hypothalamus and olfactory tubercle, and simultaneously decreases in cortical and spinal regions (Gimpl and Fahrenholz, 2001). ERα cells are detected in the rat brain by E16 and within the MPOA by E18 (DonCarlos, 1996; Al-Bader et al., 2008). ER mRNA levels in the MPOA of rats are not different between males and females until birth. However, there is an effect of intrauterine position, such that being positioned between two females increases MPOA ER mRNA relative to being positioned between two males (the two ER isoforms were not differentiated here; DonCarlos, 1996). There is a widely studied critical period for estrogen to masculinize sexually dimorphic brain region morphology, including the MPOA, from E18 to postnatal day 5, which is consistent with timing of ER development (Rhees et al., 1990b; Rhees et al., 1990a; DonCarlos and Handa, 1994; Schwarz and Mccarthy, 2008). The distribution of ERα-expressing cells is similar among neonates and adults (Yokosuka et al., 1997), although

levels increase significantly in the hypothalamus and amygdala during puberty (Brown et al., 1994). Hypothalamic ER binding levels are similar in female rats through young and middle adulthood (2.5 to 10 months), and decline again with increasing age (19 months; Brown et al., 1990). While studies have defined the developmental time course of sex-differences within the neuroendocrine system (Schwarz and Mccarthy, 2008), it was previously unknown when individual variation in female ERα levels emerge.

Early life experiences are able to influence individual variation in adult hormone receptor levels in the MPOA and hypothalamus. For example, male rats exposed to maternal separation have lower OTR binding in the lateral septum and higher OTR binding in the MPOA and ventral medial hypothalamus compared to control siblings (Lukas et al., 2011). Female mice that experience communal rearing by three dams compared to a single dam have elevated OTR binding in the LS as adults (Curley, 2009), and prairie voles reared biparentally have higher oxytocin mRNA in the paraventricular nucleus of the hypothalamus compared to those reared by a single mother (Ahern and Young, 2009). Likewise, hormone receptor variation in adult offspring is associated with the level of maternal LG received by rats. Adult virgin female offspring reared by Low LG dams have lower levels of estradiol-induced OTR binding (Champagne et al., 2001) and ERα mRNA and protein (Champagne et al., 2003b; Champagne et al., 2006) in the MPOA compared to adult virgin female offspring of High LG dams. Differences in OTR binding between High and Low LG female offspring are eliminated with ovariectomy, and estrogen replacement only reinstated increased OTR binding in High LG females, indicating that receptor levels are stable (Champagne et al., 2001). The studies described in this thesis aimed to understand the developmental time course and regulation of these neuroendocrine changes in response to maternal care.

Mesolimbic dopamine pathways

Maternal motivation

In addition to being hormonally driven, maternal behavior is also a motivated behavior, and as such involves the mesolimbic dopamine system well known for its role in reward processing and motivated behaviors. In thinking about maternal behavior in the framework of a motivated behavior, we can examine both appetitive and consummatory behaviors. Appetitive aspects of maternal behavior are the motivated drives preceding active maternal care (Lonstein and Morrell, 2006). Appetitive drives are complex: to approach a pup a subject must overcome any anxiety about the environment and/or pup, and the pup must be sufficiently incentivizing. Consummatory behaviors are the outwardly expressed behaviors including pup retrieval to a nest, LG, and nursing (Lonstein and Morrell, 2006). Often motivated behaviors are defined by a subject's willingness to do work to gain access to the stimulus, and maternal motivation has been well illustrated by several studies. In order to gain access to pups, lactating dams will cross electrified grids (Nissen, 1930), learn mazes (Simmons, 1924), and enter anxiety-provoking environments (Gandelman et al., 1970; Bridges et al., 1972; Stern and Mackinnon, 1976). Lactating dams have even learned to bar press for access to pups, and have been reported to persist in bar pressing and pup retrieval for hours (Winsoncroft, 1969; Hauser and Gandelman, 1985). Virgin and ovariectomized females, on the other hand, will retrieve significantly fewer pups, indicative of the interactions between hormonal and motivational brain systems necessary for maternal care (Hauser and Gandelman, 1985). Studies have also examined the relative incentive value for pups compared to other highly rewarding stimuli in conditioned place preference tests (CPP), in which the subject learns to associate a stimulus (e.g. pups) with a place

(e.g., one chamber of a multi-chambered testing apparatus with unique visual/textural cues). Dams tested for their preference for pups *vs.* cocaine in the early postpartum period (day 8) prefer pup-associated chambers, while dams conditioned and tested later in the postpartum period (day 16) prefer cocaine (Mattson et al., 2001; Wansaw et al., 2008). The early motivation for pups is due to a combination of hormonal state and changing salience of pups as they age, as early postpartum dams prefer pups of any age, while late postpartum dams will prefer pups compared to cocaine, but only if the pups are young (less than one week old) (Wansaw et al., 2008). However, these changing preferences are stimulus-specific rather than due to global motivation differences across lactation, as both early and late lactating dams prefer cocaine when compared with saline (Seip et al., 2008). Together these studies demonstrate that pups are highly incentivizing to lactating dams, primed by their hormonal state.

Mesolimbic dopamine system: brain regions and signaling

The mesolimbic dopamine system includes the main neural substrates underlying motivated behaviors (Kelley and Berridge, 2002; Wise, 2002). This pathway includes dopamine neurons in the ventral tegmental area (VTA) that project to limbic regions including the nucleus accumbens (NAc, particularly the shell region) of the ventral striatum, amygdala, hippocampus, and medial prefrontal cortex (Beckstead et al., 1979; Björklund and Dunnett, 2007; Ikemoto, 2007; Sesack and Grace, 2009). These regions were originally identified by studies in which animals would learn to work for direct electrical stimulation of the brain region, and molecularly defined by studies in which animals would self-stimulate with direct drug injections (see Wise, 2002). Activation of dopamine neurons in the VTA and release of dopamine within target regions is the classic mechanism of reward or motivation. GABAergic neurons are also

prominent in the VTA and normally inhibit local dopaminergic neurons (Johnson and North, 1992; Sesack and Grace, 2010). $GABA_A$ antagonists are self-administered to the VTA (Ikemoto et al., 1997), and opiates have been shown to have their rewarding effects via inhibition of GABAergic neurons through mu and delta receptors, and thus act by disinhibition of dopamine neurons (Johnson and North, 1992; Wise, 2002). The VTA receives afferent projections from a number of brain regions, including glutamatergic inputs from the prefrontal cortex and hormone responsive neurons from the MPOA and other hypothalamic regions, discussed in further detail below.

Medium spiny neurons of the NAc receive a main dopaminergic efferent from the VTA that is transduced by dopamine receptors (Bouyer et al., 1984). Dopamine receptors are GPCRs that are classed into D1-like and D2-like receptors: D1-like receptors include dopamine receptors D1 and D5, while D2-like receptors include receptors D2, D3, and D4 (Beaulieu and Gainetdinov, 2011). D1-like receptors activate $G\alpha_{-s/olf}$ family GPCRs to stimulate adenylyl cyclase, cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA) signaling (Beaulieu and Gainetdinov, 2011). D1-like receptors are found post-synaptically on medium spiny neurons in the NAc (Beaulieu and Gainetdinov, 2011). D2-like receptors couple with $G\alpha_{-i/o}$ family proteins to inhibit adenylyl cyclase. D2 and D3 receptors can be found both pre-synaptically, as autoreceptors involved in negative feedback and regulation of firing rate/ reduced dopamine release, and post-synaptically on medium spiny neurons of the NAc (Sokoloff et al., 2006; Beaulieu and Gainetdinov, 2011). Two splice variants of the D2 receptor have been identified, S and L. The D2S variant is predominantly a presynaptic, while the D2L variant is predominantly expressed postsynaptically (Beaulieu and Gainetdinov, 2011). A small population of NAc neurons expresses both D1 and D2 neurons (Surmier et al., 1996). D1 and

D2, and to a lesser extent, D3 receptors are critically involved in motivated and reward-directed behaviors (Self et al., 1996; Kelley and Berridge, 2002; Beaulieu and Gainetdinov, 2011).

Development of the mesolimbic dopamine system

Development of the midbrain dopaminergic regions is complex with many contributing drivers, including a series of transcription factors (see Prakash and Wurst, 2006). Tyrosine hydroxylase (TH) is the first and rate limiting enzyme in the dopamine synthesis pathway, shared by norepinephrine and epinephrine, and is a marker of dopaminergic neurons. TH-immunoreactivity is first observed in the developing rodent midbrain at embryonic day 10.5 (Riddle and Pollock, 2003). Two transcription factors critical for midbrain dopaminergic development are Nurr1 (Nr4a2) and cyclin-dependent kinase inhibitor n1c, Cdkn1c ($p57^{kip2}$). *Nurr1* encodes an orphan nuclear receptor of the ligand-activated superfamily (including estrogen receptors; Perlmann and Wallen-Mackenzie, 2004) and is dependent upon interaction with Cdkn1c to induce cell cycle arrest and dopamine neuron differentiation (Joseph et al., 2003). *Nurr1* transcription is found as early as E10.5 in mice, when midbrain dopaminergic neurons are terminally differentiating, and continues to be expressed in the postnatal period. *Cdkn1c* may be under transcriptional control of Nurr1, is expressed in differentiating midbrain dopaminergic precursors, and *Cdkn1c* knockouts phenocopy *Nurr1* null mice (Joseph et al., 2003). *Cdkn1c* knockout mice have increased apoptosis and delayed differentiation (Yan et al., 1997), highlighting the importance of these transcription factors in dopamine neuron differentiation. Additionally, the *Th* promoter contains specific Nurr1 recognition sites and Nurr1 can directly transactivate *Th* in some cell lines (Sakurada et al., 1999; Kim et al., 2003). Lmx1b (of the LIM homeodomain family) and Pitx3 (paired-like homeodomain transcription

factor) are two transcription factors implicated in midbrain dopamine neuron survival and maintenance. *Lmx1b* null mice were initially able to generate TH-expressing neurons but the neurons do not survive and *Th* is undetectable after E16 (Smidt et al., 2000). Pitx3, widely expressed in development but restricted in adulthood exclusively in the VTA and SN, is also diminished in *Lmx1b* mutant mice (Smidt et al., 2000; Smidt, 2004). *Lmx1b* and *Pitx3* are thought to represent a second pathway for dopamine neurons differentiation and maintenance, independent of *Nurr1* and *Cdkn1c*, because *Lmx1b* is normally expressed in *Nurr1* mutant mice (Smidt et al., 2000; Prakash and Wurst, 2006). Maturation of midbrain dopaminergic neurons continues through the third postnatal week when the distribution and morphology of dopaminergic fibers in the striatum attains adult characteristics (Voorn et al., 1988).

Table 1.1 Transcription factors implicated in mesolimbic dopamine system development

Transcription Factor	Function in mesolimbic dopamine development
Nurr1 (Nr4a2)	orphan nuclear receptor; transactivats *Th*; with Cdkn1c, induces cell cycle arrest and dopamine neuron differentiation; knockout mice have delayed dopamine neuron differentiation, failure of dopamine neurons to innervate striatal targets, and increased apoptosis
Cdkn1c ($p57^{kip2}$)	is transcriptionally activated by and binds with Nurr1 to induce cell cycle arrest and dopamine neuron differentiation; knockout mice phenocopy *Nurr1* knockout mice
Lmx1b	maintenance of midbrain dopamine neurons; widely expressed in development but found exclusively in the VTA and SN in adulthood; normally expressed in *Nurr1* knockout mice; *Lmx1b* knockout mice have reduced Pitx3 and dopamine neurons differentiate but are not maintained
Pitx3	survival and maintenance of midbrain dopamine neurons; transcriptionally activated by Lmx1b; found exclusively in the VTA and SN in adulthood; colocalizes with TH in subsets of midbrain doapmine neurons; mice lacking Pitx3 have increased apoptosis, significantly fewer dopamine neurons in the VTA, and reduced innervation of targets

The mesolimbic dopamine system in maternal behaviors

Activation of the VTA and NAc is associated with maternal behavior. Lactating dams stimulated by pup suckling in fMRI show enhanced BOLD signal in the VTA and NAc, among other regions (Febo, 2005), and increased dopamine release is found in the ventral striatum of lactating dams after being separated and reunited with their litter (Hansen et al., 1993).

Inhibiting dopamine signaling in these regions disrupts maternal care. Lesions severing tracts between the MPOA and VTA greatly diminish maternal behaviors (Numan and Smith, 1984), as do electrolytic and 6-OH-DA lesions in the VTA or NAc (Gaffori and Le Moal, 1979; Hansen et al., 1991). Baclofen injections into the VTA inhibit maternal retrieval behavior (but not pup investigation) by stimulating $GABA_B$ receptors and depressing dopamine release in the NAc (Westerink et al., 1996; Numan and Stolzenberg, 2009). Inhibition of dopamine receptors also inhibits maternal behavior. Systemic haloperidol, a mixed D1/D2-like antagonist, dose-dependently inhibited pup retrieval and LG, but not food-seeking behavior (Giordano et al., 1990; Stern and Taylor, 1991). Similarly, direct blockade of NAc D1 and D2 receptors by flupenthixol was found to dose-dependently inhibit maternal retrieval and LG behaviors but enhance nursing (if the dam was placed over the pups), indicating that VTA-NAc signaling may be selectively involved in appetitive maternal behaviors (Keer and Stern, 1999). D1 receptors in the NAc may be of greater importance to maternal behavior, as D1 (but not D2) antagonists injected directly into the NAc of postpartum dams disrupt pup retrieval (Numan et al., 2005).

Activation of the mesolimbic dopamine system also promotes the onset of maternal behaviors in hormonally primed rats. Pharmacological activation of NAc D1 receptors (linked with adenylyl cyclase signaling, but not D1 receptors linked with phospholipase C, PLC, signaling) was able to enhance maternal sensitization and pup retrieval in pregnancy-terminated

female rats (Stolzenberg et al., 2010). The necessity of hormonal priming for the mesolimbic dopamine system to be responsive to pup stimuli is further demonstrated by increased dopamine release in the NAc shell in response to pups only in postpartum and hormone-treated females, but not control females (Afonso et al., 2009). Because the mesolimbic dopamine system is integrally involved in response to a multitude of natural rewards and stimuli, differential activation of neuroendocrine systems is one potential mechanism for modulating the incentive salience of pups and motivated maternal behaviors.

In support of this theory, individual differences in maternal behavior have been linked to variation in the mesolimbic dopamine system in addition to variation in hypothalamic OTR and ERα. High compared to Low LG dams have greater numbers of oxytocin neuron projections from the MPOA and PVN to dopamine neurons of the VTA (Shahrokh et al., 2010). Preceding and during bouts of LG there is greater dopamine release in the shell of the NAc in High compared to Low LG dams, indicating this signal in the appetitive aspects of maternal behavior, and the duration of the signal increase is highly correlated with the duration of the LG bout (Champagne et al., 2004). High compared to Low LG dams have greater dopamine transporter and dopamine receptor D1 and D3 binding in the NAc shell (Champagne et al., 2004). Treatment with dopamine uptake inhibitors increased the dopamine signal and duration of LG bout in Low LG dams to levels comparable to High LG dams (Champagne et al., 2004). However, these variations in mesolimbic signaling have only been explored in adult and lactating dams, and it was previously unknown whether variation within this system was influenced developmentally by the quality of maternal care.

The mesolimbic dopamine system is not considered fully mature until the third postnatal week in rodents (Voorn et al., 1988), and there is evidence that prenatal and postnatal

experiences shape the mesolimbic dopamine system within this period. Many studies have shown that stressful experiences in the prenatal and early postnatal period alter dopaminergic function (Alonso et al., 1994; Henry et al., 1995; Ortiz et al., 1996; Barros et al., 2004; Mcarthur et al., 2005; Jahng et al., 2010; Rodrigues et al., 2011; Baier et al., 2012; Huppertz-Kessler et al., 2012; Ventura et al., 2012). The effects of prenatal stress in rodents include increased dopamine release and decreased metabolites in the nucleus accumbens (NAc) in adulthood (Alonso et al., 1994), increased dopamine cells in the VTA and SN in adulthood (Mcarthur et al., 2005; Mcarthur et al., 2007), increased dopamine receptor type 2 (D2) binding and decreased D3 binding in the NAc (Henry et al., 1995). Adoption of rat pups at birth has been shown to be sufficient to reverse a prenatal stress-induced increase in D2 receptor binding in the prefrontal cortex and hippocampus (Barros et al., 2004), indicating that the dopamine system continues to be sensitive to environmental experiences in the early postnatal period. Early postnatal stressors such as glucocorticoid administration or maternal separation in rodents increased the number of dopamine cells in the VTA and SN (Mcarthur et al., 2005; Mcarthur et al., 2007; Chocyk et al., 2010, in an age- and sex-dependent manner), increased striatal dopamine (Huppertz-Kessler et al., 2012), but decreased dopamine transporter binding in the striatum (Meaney et al., 2002). Postnatal motherless rearing in rats increases basal NAc dopamine but increases or decreases dopamine response to stimuli in a stimulus-specific manner (Afonso et al., 2011; increased response to food, decreased response to pups), and an unstable maternal environment reduces preference for palatable food stimuli (Ventura et al., 2012). The effects of postnatal handling, thought to ameliorate stress response in adulthood (Liu et al., 2000b; Plotsky et al., 2005) include increased baseline NAc dopamine (Silveira et al., 2010) and decreased NAc dopamine release and D3 binding in response to stress (Brake et al., 2004). Together these studies highlight that

both molecular and behavioral alterations to the dopamine system during early development can last well into adulthood. While these studies have focused on stressful manipulations, little is known about how natural variation in the quality of the early maternal environment influences offspring dopamine system development.

Estrogen and oxytocin interactions with the mesolimbic dopamine system

The hypothalamic neuroendocrine and mesolimbic dopamine systems have been shown to interact, particularly in the context of maternal and sexual behaviors. Interactions are found both at the anatomical level as well as the molecular level. Sensitivity of the VTA and NAc to estrogen has been explored partially by studies of ovariectomy and estrogen replacement. However, these studies have generated inconsistent findings that vary depending on the length of time since ovariectomy, whether estrogen was given by implanted capsule or daily injections, and whether the effects of estrogen treatment are being compared to intact or ovariectomized animals. The results of some of these studies are summarized in **Table 1.2**. Treatment of ovariectomized females with estradiol increased firing probability in dopamine neurons of the VTA, providing strong physiological evidence for midbrain sensitivity to estrogen (Sakamoto et al., 1993). Another study of particular interest examined the number of TH-immunoreactive (-ir) cells in the VTA and SN of ovariectomized mice and rats and found that the number of dopamine neurons were reduced in both midbrain regions, in both species, compared to intact animals (Johnson et al., 2010). Furthermore, treatment for two weeks with implants containing estradiol or an ERα agonist normalized the level of Th-ir cells in the VTA of rats, while ERß treatment did not significantly alter the level of TH-ir cells in the VTA (though ERß treatment was found to normalize TH-ir cells in the SN of rats, as well as in the SN and VTA of mice;

Johnson et al., 2010). Alterations in the midbrain dopamine system were also detected in estrogen receptor knockout mice: compared to controls, both female and male mice lacking ERα had decreased brain-derived neurotrophic factor (BDNF) and TH mRNA and protein levels in the ventral midbrain (Kuppers et al., 2008), and decreased TH-ir cells in the VTA (Johnson et al., 2010). Interestingly, however, the number of TH-ir cells in the VTA of mice lacking ERß was not different from wild-type controls, suggesting that the effects of estrogen on TH are mediated through ERα (Johnson et al., 2010).

Table 1.2 Effects of ovariectomy and estrogen replacement on dopamine system components.

Region	Target	Compared to Intact	Compared to OVX	Ovariectomy		Effect of estrogen therapy		Reference
				Long-term	Short-term (<2 wks)	implanted capsule	Injections	
VTA	TH-immunoreactivity	✓		↓		↑, ERα-dependent		Johnson et al., 2010
	TH mRNA		✓	✓	✓	=	↑↓ short-term, long-term	Serova et al., 2002
	TH mRNA, primate		✓		✓		↑ single	Pau et al., 2000
	DAT mRNA	✓		↑	=	=		Bosse et al., 1997
	D2/D3 binding		✓		✓	↓		Febo et al., 2003
NAc / ventral striatum	DAT binding	✓		↓	=	↑		Bosse et al., 1997
	D2/D3 binding		✓		✓	=		Febo et al., 2003
	DAT binding	✓		↓		↑		Chavez et al., 2009
	D1 binding	✓		=		=		Chavez et al., 2009
	D2 binding	✓		↑		↓		Chavez et al., 2009
Nucleus of the solitary tract	TH mRNA		✓		✓	↓	↑	Sabban et al., 2009
Locus Coeruleus	TH mRNA		✓		✓	=	=	Sabban et al., 2009

Molecular interactions between estrogen and the dopamine system have been observed in culture. Estrogen induces dopamine efflux from cells *in vitro* via the dopamine transporter

(DAT), and is dependent upon PKC activation but not PKA or PI3K kinases (Alyea and Watson, 2009). siRNA knockdown of ERα but not ERß abolished the estrogen-mediated dopamine efflux, and treatment with an ERß agonist showed ERß to have an inhibitory effect on dopamine efflux (Alyea et al., 2008). In cells that express ERα but not ERß, estradiol treatment increases TH promoter activity, as measured by luciferase, while estradiol decreases TH promoter activity in cells the express ERß but not ERα (Sabban et al., 2010). These results, together with the knockout studies, indicate the possibility for estrogen to directly regulate dopamine neurons via ERα. Paradoxically, however, ERß, and not ERα, has been found in the VTA and SN of rodents (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001). Evidence also suggests that dopamine can reciprocally affect ERα in a ligand-independent manner. Dopamine acting through D1 receptors activates PKA and cAMP signaling pathways, which can stimulate ERα activation (Gangolli et al., 1997; Riby et al., 2000; Al-Dhaheri and Rowan, 2007).

Neurons of the MPOA, BNST, and anterior hypothalamic area project to the VTA (Morrell et al., 1984). Stimulation of the MPOA increases VTA dopamine neuron firing probability and lesioning the MPOA decreases VTA firing probability in female rats (Sakamoto et al., 1993). MPOA and BNST neurons projecting to the VTA were found to be estrogen-concentrating (likely expressing ERα and/or ERß and responsive to estradiol, but not tested directly for the receptors in the study) (Morrell et al., 1984). The estrogen-responsive VTA projecting neurons of the MPOA are likely to be oxytocinergic, as nearly half of the estrogen-concentrating neurons of the MPOA were found to have oxytocin in their cytoplasm and oxytocin neurons of the MPOA were found to project to the VTA (Morrell et al., 1984; Shahrokh et al., 2010). However, labeling for all three factors has not been done simultaneously, and it is not known whether oxytocin neurons synapse directly onto dopamine neurons, GABAergic

neurons, or both. A small population of MPOA neurons projecting to the VTA has also been identified as glutamatergic (Geisler et al., 2007). In addition, GABAergic neurons projecting from the MPOA terminate in the caudal tail of the VTA, which has a low concentration of dopamine neurons and a high concentration of locally synapsing GABA neurons (Kaufling et al., 2009). Together these studies suggest that, in the developed brain, ERα neurons of the MPOA likely exert an effect on the dopaminergic system indirectly via oxytocin and glutamate stimulation and via disinhibition of dopamine neurons (release from GABAergic suppression; **Figure 1.3**).

Figure 1.3

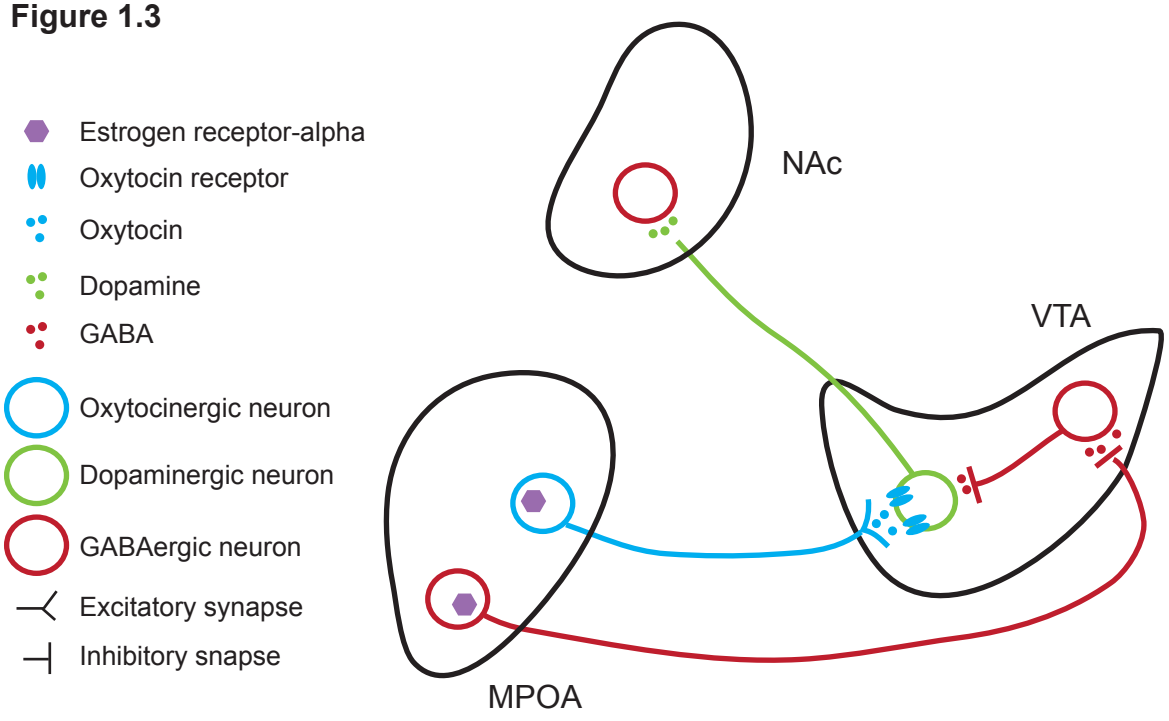


Figure 1.3 Schematic of the hypothesized circuitry between estrogen-sensitive neurons of the MPOA and dopaminergic neurons of the VTA

Oxytocinergic and GABAergic neurons of the MPOA have been shown to project to different regions of the VTA and are likely estrogen-sensitive. Oxytocin projections are excitatory and dopamine neurons are likely to directly stimulate dopamine neurons. GABAergic projections from the MPOA are likely to disinhibit dopaminergic neurons of the VTA. One main dopaminergic projection from the VTA is to medium spiny neurons of the NAc.

Consistent with the idea that estrogen exerts an effect on the VTA indirectly via oxytocin release, oxytocin receptor binding has been described in the VTA (Pedersen et al., 1994). Oxytocin infused into the VTA increases dopamine output signal in the NAc of virgin females (Shahrokh et al., 2010). Oxytocin receptor antagonists applied directly in the VTA of lactating dams have been found to decrease fMRI BOLD signal induced by pup suckling and by oxytocin (Febo, 2005) and decrease dopamine signal in the NAc (Shahrokh et al., 2010). Behaviorally, antagonists decrease pup retrieval by lactating dams (Pedersen et al., 1994). Variation in MPOA-VTA oxytocin projections and oxytocin-induced dopamine release has also been observed in Low and High LG dams. Greater numbers of oxytocin neurons were found to project from the MPOA and PVN to the VTA in lactating High compared to Low LG dams (Shahrokh et al., 2010). There was also greater peak dopamine signal in the NAc shell during pup LG in High compared to Low LG dams, and that difference was eliminated by infusion of an oxytocin antagonist directly into the VTA (Shahrokh et al., 2010). These anatomical variations appear to underlie signaling and behavioral differences. However, the development of these differences, including determination of whether the variation observed in the dopaminergic system of females is independent or a direct consequence of variation in the hypothalamic neuroendocrine system had not previously been explored.

Developmental interactions of the estrogen system with midbrain dopamine development have been suggested. While ERα is not found in the adult VTA, ERα and ERß were transiently expressed in developing dopaminergic populations from E17 to PN10, as was aromatase, indicating that locally converted estrogen may directly influence developing dopamine cells (Raab et al., 1995; Kritzer, 1997; Simerly et al., 1997; Karolczak et al., 1998; Christian and Gillies, 1999; Raab et al., 1999; Kuppers et al., 2001). Importantly, this developmental period of

midbrain aromatase and estrogen receptor expression continues through the early postnatal period and its actions and effects may therefore: 1) have long-lasting organizational effects, and 2) be influenced by early life experiences (Kipp et al., 2006). *In vitro*, treatment of dopamine neurons from embryonic mice with estradiol stimulated BDNF and GDNF via membrane-bound ERα, and increased neurite growth and arborization in a PKA-dependent manner (Beyer et al., 2003; Kipp et al., 2006). Cultured embryonic midbrain slices treated with estradiol also had elevated TH mRNA and protein levels (Ivanova et al., 2001; Ivanova and Beyer, 2003). Estrogens and ER activity have also been shown to have life-long neuroprotective effects, particularly within the cortex and hippocampus (see (Behl, 2002) as well as in the developing midbrain (Kipp et al., 2006). Estrogens may mediate neuroprotective effects in the developing midbrain via the antiapoptotic PI3K/Akt pathway (Ivanova et al., 2001; Kipp et al., 2006). These studies outline several mechanisms by which estrogen and estrogen receptors may participate in mesolimbic dopamine neuron development and maintenance, although it was not known whether there is variation in these systems among Low and High LG dams or their offspring.

Epigenetic regulation of gene expression by DNA methylation and post-translational histone modifications

In attempting to understand how complex adult behaviors may be influenced by early life experiences, we must understand mechanisms involved in long-term regulation of neuronal systems and gene expression. Gene expression can be regulated through epigenetic mechanisms, which can be stable or dynamic. The term ‘epigenetic’ refers to chromatin modifications that alter gene expression without affecting DNA sequence. Epigenetic mechanisms of gene

regulation include DNA methylation, post-translational histone protein modifications, and effects on transcription/translation via small RNAs (microRNAs; see **Figure 1.4**) (Razin, 1998; Bird, 2002; Saha et al., 2006; Schaefer et al., 2007; Ooi and Bestor, 2008b; Riccio, 2010; Sharma et al., 2010).

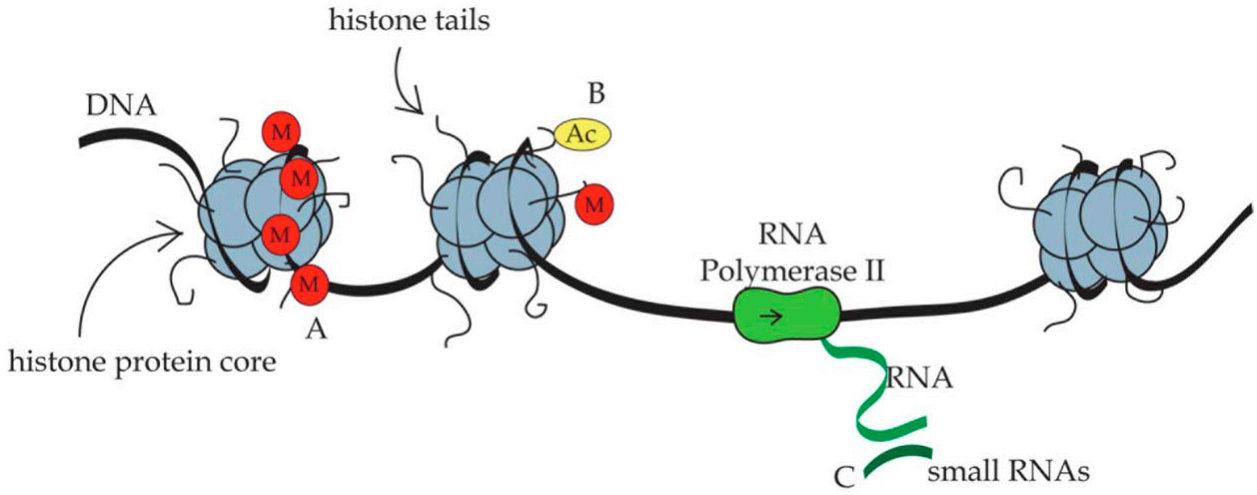


Figure 1.4 Epigenetic regulation of gene expression

DNA is wrapped around histone proteins and gene expression is dependent on the enzyme RNA Polymerase II accessing the DNA and creating RNA. (A) Attachment of methyl chemical groups (M) to the DNA sequence suppresses gene expression. (B) Histone proteins have "tails" that can be modified by attachment of acetyl (Ac) and methyl groups to specific sites. Histone tail acetylation increases gene transcription, while histone tail methylation typically decreases gene activity. (C) Small RNA molecules such as microRNAs can bind to and degrade newly transcribed RNA, repressing gene activity.

DNA methylation

DNA methylation is one way in which the structure of DNA can be altered by chemical covalent attachment of methyl groups to cytosine-guanine nucleotide (CpG) pairs to affect transcription without altering the sequence of the gene. DNA methylation is thought to be more stable than other epigenetic marks because of its covalent bond, and the level of DNA

methylation is therefore traditionally thought to be maintained through cell division and across the lifespan (Razin, 1998; Bird, 2002; Saha et al., 2006; Schaefer et al., 2007; Ooi and Bestor, 2008b; Riccio, 2010; Sharma et al., 2010). CpG dinucleotides typically cluster within or around gene promoters in "CpG islands" that tend to be hypomethylated in normal somatic cells (Bird and Wolffe, 1999). Gene promoter hypermethylation prevents access of transcriptional activators and RNA polymerase II to the transcription start site and is associated with decreased gene expression (Razin, 1998). The process of methylation is dependent on the presence of methyl donors (provided by nutrients such as folic acid, methionine and choline) and DNA methyltransferases which mediate either maintenance (i.e. DNMT1) or *de novo* DNA methylation (i.e. DNMT3). Transcriptional repression associated with DNA methylation is further sustained through methyl-binding proteins such as MeCP2 (Fan and Hutnick, 2005). DNA methylation is also known work in concert with histone modifications to further silence gene transcription (Fuks, 2005; Klose and Bird, 2006).

Chromatin remodeling

Histone protein modifications represent more dynamic process of gene regulation. DNA is compactly folded into chromatin with the help of an octomer of histone proteins (H2A, H2B, H3, and H4, each represented twice) in order to store and maintain the integrity of the DNA and to regulate gene expression. When chromatin is tightly coiled (euchromatin), gene expression is repressed. In contrast, when chromatin is in an open configuration (heterochromatin), transcription factors and transcriptional machinery such as RNA polymerase II have access to the DNA and can initiate gene transcription. Chromatin conformation can be altered by post-translational modifications to the histone proteins. N-terminal tails from the histones protrude

from the nucleosome core and may be modified at specific sites by acetylation, methylation, ubiquitination, and phosphorylation (**Figure 1.5**). Histone acetylation is associated with increased transcriptional activity, whereas histone deacetylation is associated with transcriptional repression. The acetylation state of these nucleosomal proteins is controlled by the presence of histone acetyltransferases (HATs), histone deacetylases (HDACs), which are recruited by methyl-binding proteins, and by HDAC inhibitors, which effectively increase gene expression by shifting histones to an acetylated state (Khorasanizadeh, 2004). Histone tails can also be methylated by histone methyltransferases (HMTs). The exact location and degree of histone tail methylation (mono-, di- or tri-methylation) either promotes euchromatin or heterochromatin, and are thus associated with either gene transcription or transcriptional repression, respectively (Ha et al., 2011). Although a complete readout of the "code" of histone marks and their relationship to transcription eludes us, there is strong evidence for individual marks influencing gene expression (Jenuwein and Allis, 2001). For example, histone 3 lysine-4 (H3K4), H3K36, and H3K79 di- and tri-methylation are associated with active gene transcription, while methylation of H3K9, H3K27, and H4K20 promote more tightly coiled heterochromatin and are associated with gene silencing (Rea et al., 2000; Jenuwein and Allis, 2001; Khorasanizadeh, 2004; Martin and Zhang, 2005; Wysocka et al., 2006; Shahbazian and Grunstein, 2007). **Table 1.3** provides a summary of some of these associations. Although epigenetic mechanisms are complex and our knowledge of the dynamics of these pathways is still in its infancy, emerging evidence suggests that epigenetic mechanisms provide a link between the experience of variation in early environmental experiences and persistent changes in gene activity, neurodevelopment, and behavior.

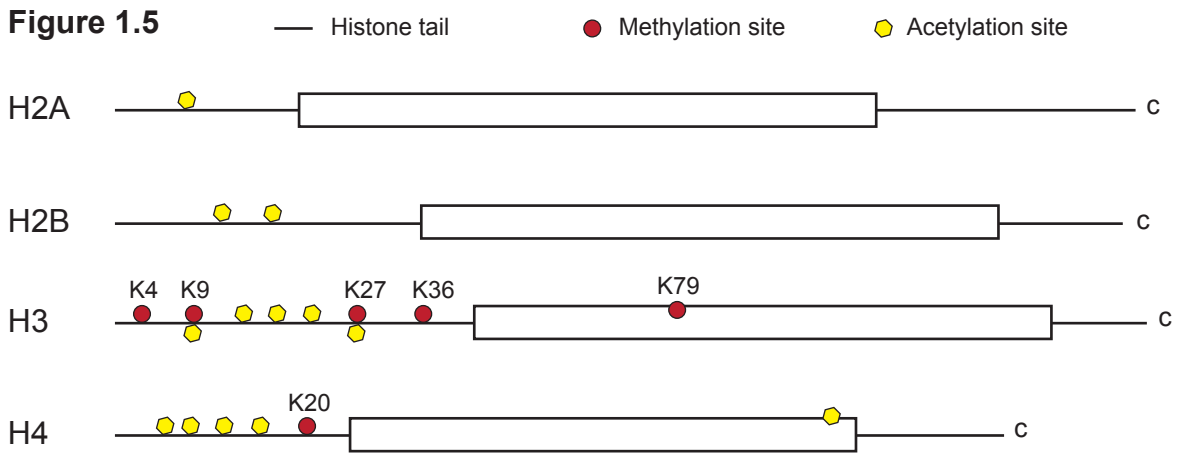
Table 1.3 Transcriptional state associated with histone tail methylation

Tri- and di-methylation at these sites are typically associated with the transcriptional state indicated. However, it should be noted that mono-methylation and sometimes di-methylation is associated with the opposite transcriptional state.

Histone Lysine Residue	Methylation associated with transcription	Methylation associated with transcriptional repression
H3K4	✔	
H3K9		✔
H3K27		✔
H3K36	✔	
H3K79	✔	
H4K20		✔

Figure 1.5 Post-translational histone protein modifications

Sites of methylation and acetylation on the four core histone proteins, adapted from Shahbazian & Grunstein, *Ann Rev Biochem*, 2007.



Epigenetic regulation of estrogen receptor-alpha

The ERα gene, *Esr1*, is known to be regulated by epigenetic mechanisms, particularly in the context of cancer. A large proportion of breast cancer cells have low levels of ERα and hypermethylation of the *Esr1* promoter (Fabianowska-Majewska et al., 2006; Giacinti et al., 2006; Zhao et al., 2009). Treatment of cultured cancer cells that also have low levels of ER expression with a DNMT inhibitor and an HDAC inhibitor restores ER levels, demonstrating dynamic regulation of *Esr1* by epigenetic mechanisms (Fan et al., 2006; Walton et al., 2008; Wei et al., 2008).

Evidence suggests that *Esr1* methylation changes with age and in response to the environment. *Esr1* methylation levels have been analyzed across early postnatal development in mouse cortex through PN25. Percent methylation was found to increase with age at every CpG site in the mouse proximal A promoter in both female and male mice, although these developmental increases were not directly studied in relation to individual differences in environmental experience or behavior (Westberry et al., 2010). ERα has been shown to protect against cell death after ischemic injury (Dubal et al., 1998). ERα is normally present at low levels in adult brain cortex, but increases dramatically after injury, an effect accompanied by dramatic reduction in *Esr1* promoter methylation (Westberry et al., 2008).

Variation in postnatal maternal care is also associated with differences in *Esr1* methylation. The rat promoter B is 87% homologous to the human *Esr1* distal B promoter (Freyschuss and Grandien, 1996; Kos, 2001) and has been shown to be used in transcription within the brain (Schibler and Sierra, 1987). Transcription from this promoter includes a 5' untranslated region immediately adjacent to the B promoter, and thus this regulatory region is termed "B/1b" (GenBank X98236.1; named as in Freyschuss and Grandien, 1996). This region

also contains response elements for Stat5b transcription factor binding, which stimulates *Esr1* transcription (Frasor and Gibori, 2003). Increased CpG methylation and decreased Stat5b binding to the *Esr1* B/1b regulatory region were found in Low compared to High LG adult female offspring, concurrent with lower ERα mRNA among adult Low LG offspring (Champagne et al., 2006). Simulated maternal grooming (additional paintbrush stimulation of the anogential region, performed PN5-7) also increases rat *Esr1* promoter B/1b methylation compared to control females at two CpG sites, as does neonatal treatment with estradiol benzoate (Kurian et al., 2010; Schwarz et al., 2010). Together these studies provide evidence that the *Esr1* gene can be epigenetically regulated in response to early life experiences. However, the time course whereby early life experience of maternal LG induces epigenetic changes to regulate *Esr1* was previously unknown.

Epigenetic changes in response to environmental events

There is an expanding literature providing examples of environmentally-induced epigenetic alterations (Fagiolini et al., 2009). Epigenetic alterations have been detected in response to prenatal environmental events within fetal tissue and adult brains. Maternal exposure to stress during gestation was associated with altered mRNA and DNA methylation levels of 11 β hydroxysteroid dehydrogenase type 2 (HSD11B2), an enzyme that buffers the impact of maternal glucocorticoid exposure by converting cortisol/corticosterone into inactive metabolites (Jensen Peña et al., 2012). In the placenta, prenatal stress was associated with a decrease in HSD11B2 mRNA, increased mRNA levels of DNMT3a, and increased DNA methylation (by 2-7%) at specific CpG sites within the HSD11B2 gene promoter, while within the fetal hypothalamus, although there were no stress-induced effects on HSD11B2 mRNA

levels, prenatal stress induced decreased CpG methylation within the HSD11B2 promoter and increased methylation at sites within exon 1 (Jensen Peña et al., 2012). Decreased DNA methylation of the corticotrophin-releasing-factor (CRF) gene promoter and increased methylation of the glucocorticoid receptor (GR) exon 1_7 promoter region were also found within the hypothalamus of adult male mice born to gestationally stressed females (Mueller and Bale, 2008). The nutritional environment during fetal development has likewise been demonstrated to influence growth, metabolism, and brain development and there is increasing evidence that dietary levels of methyl-donors can epigenetically alter gene expression in offspring (Hoet and Hanson, 1999; Zeisel, 2009). In rats, GR exon 1_{10} methylation is reduced in the hepatic tissue of offspring born to protein restricted dams whereas methylation is increased in offspring of dams whose diet is supplemented with methyl donors (Lillycrop et al., 2007; Lillycrop et al., 2008). These effects may be related to DNMT1 expression, which is likewise decreased with dietary protein restriction (Lillycrop et al., 2007). Intrauterine growth restriction by maternal food restriction or low-protein diet during gestation also resulted in chromatin remodeling, including altered H3K4 methylation association with several genes important for growth and tumor suppression (Tosh et al., 2010; Jousse et al., 2011; Sohi et al., 2011; Zheng et al., 2011).

The postnatal environment has also been found to induce epigenetic alterations within the brain. CRF actions are potentiated by arginine vasopressin (AVP), which was found to be hypomethylated in response to early life stress in mice (Murgatroyd et al., 2009). Altered AVP promoter methylation was sustained across the lifespan at six weeks, three months, and 12 months of age, but was also found to be responsive to treatment with the DNMT inhibitor 5-azacytadine in culture (Murgatroyd et al., 2009). Early life stress induced by maternal separation from PN2-9 decreased total histone H3K9 mono and trimethylation in the frontal cortex of mice,

but association of these epigenetic marks with specific gene targets was not explored (Kao et al., 2012). Maternal separation has also recently been shown to decrease mRNA levels of several HDACs in the cortex, an effect accompanied by increased levels of H4K12 acetylation, and specific to Balb/C but not C57Bl/6 mice (Levine et al., 2012). Analyses of levels of promoter methylation within hippocampal GR 1_7 in offspring of Low and High LG rats indicated that high levels of care are associated with decreased promoter methylation and increased GR gene expression (Weaver et al., 2004). Central infusion of a methyl donor, or conversely a HDAC inhibitor, reversed the effects of maternal LG on GR methylation, expression, behavioral responses to stress, indicative of a causal relationship among maternal LG, DNA methylation, GR gene expression, and adult stress responses (Weaver, 2005; Weaver et al., 2006). In rats, an increase in methylation of exon IV of the BDNF promoter and consequent decrease in BDNF mRNA in the prefrontal cortex was found in association with exposure to periods of abusive maternal care (dragging, rough handling, etc.; Roth et al., 2009). The effects of rat postnatal enrichment on epigenetic regulation of offspring BDNF have also been examined (Kuzumaki et al., 2011; Roth and Sweatt, 2011). Three to four weeks of enriched social and physical environments increased mouse hippocampal BDNF mRNA and H3K4me3 at the BDNF P3 and P6 promoters, while simultaneously decreasing H3K9me3 and H3K37me3 at the BDNF P3 and P4 promoters (Kuzumaki et al., 2011). As was the case with the effects of individual differences in maternal care, these effects emerged in infancy and were sustained into adulthood.

Epigenetic regulation of gene expression continues to be sensitive to environmental experiences through adulthood. In response to three weeks of caloric restriction, mice showed depressive-like behaviors and the CRF promoter was found to be hypomethylated in the BNST, but not central amygdala, showing brain region-specific regulation of this epigenetic

modification (Pankevich et al., 2010). Levels of CRF gene expression and DNA methylation were not restored by refeeding, suggesting that the adult experience of caloric restriction permanently alters that system (Pankevich et al., 2010). Acute restraint stress in adulthood was found to increase global H3K9me3 in the mouse hippocampus, particularly within retrotransposable elements, although association of H3K9me3 with specific genes in this paradigm is unknown (Hunter et al., 2012).

These findings from rodent studies are likewise mirrored by recent findings demonstrating environmentally-induced epigenetic alterations in humans. Maternal depression was found to be associated with increased GR 1F promoter methylation in fetal blood samples and these methylation patterns predicted HPA reactivity in infants at 3 months of age (Oberlander et al., 2008). Analysis of brain tissue from suicide victims with a history of childhood abuse found differential promoter methylation at 362 genes (Labonté et al., 2012). Specifically within hippocampal tissue, childhood abuse was associated with lower GR expression and higher GR 1F promoter methylation (McGowan et al., 2011). Childhood adversity was likewise associated with increased GR promoter methylation and attenuated cortisol responses in leukocytes of healthy adults, suggesting that some environmentally-induced epigenetic modifications can be detected in peripheral tissues (Tyrka et al., 2012) Convincing evidence of environmentally-induced epigenetic alterations in humans also comes from a twin study that found monozygotic twins were epigenetically indistinguishable early in life (3 years old), but among older twins (50 years old) researchers found considerable differences in the total level and distribution of DNA methylation and histone acetylation, which was associated with variation in gene expression as those loci (Fraga et al., 2005).

There is increasing evidence that environmentally-induced epigenetic alterations can be transmitted to the next generation. If altered epigenetic patterns are induced in relevant genes within the gametes, then germ-line transmission may be achieved. This phenomenon may be particularly relevant to the paternal transmission of behavioral and neurobiological outcomes. For example, recent studies have implicated transgenerational epigenetic programming through the germ line of animals exposed to the fungicide/endocrine disruptor vinclozolin (Anway et al., 2006; Crews et al., 2007; Anway et al., 2008; Nilsson et al., 2008; Skinner et al., 2008; Guerrero-Bosagna et al., 2010). Altered gene expression, significantly reduced levels of DNMT3a, and altered DNA methylation were detected within the sperm of offspring and grand-offspring of males exposed *in utero* to vinclozolin (Anway et al., 2008; Guerrero-Bosagna et al., 2010). Paternal transmission of altered epigenetic patterns was also detected among the offspring of prenatally stressed males. Both the prenatally stressed fathers and male offspring were shown to be dysmasculinized and stress-sensitive, and had altered patterns of microRNAs in the whole brain (Morgan and Bale, 2011). However, it is also possible in this case that the females mated with the gestationally stressed males were able to detect behavioral or other abnormalities, and affect offspring by differentially allocating resources (prenatal, or postnatal in the form of care; (Curley et al., 2011). This theory was tested in one study that measured maternal care of females that were mated with either males (F0) that had been stressed by chronic unpredictable maternal separation, or the F1 male offspring of stressed males (Franklin et al., 2010). Offspring in the F1, F2, and F3 generations displayed increased stress and depressive-like behavior (although this was not consistent across sexes tested), and altered methylation within the MeCP2, CB1, and CRFR2 promoters (Franklin et al., 2010), yet maternal care of females mated with F1 males was not altered, thus indicative of germ-line effects.

In addition to potential germline epigenetic inheritance of environmentally-induced epigenetic changes, there is also the possibility that maternal care (*in utero* or postpartum), through its developmental effects on epigenetic pathways in the brain, can lead to the stability of epigenetic variation across generations. For example, cross-fostered females reared by a High LG dam, regardless of the LG status of the birth mother, were found to have elevated levels of maternal LG, thought to be mediated by elevated levels of ERα mRNA in the MPOA and reduced levels of *Esr1* B/1b DNA methylation (Champagne et al., 2006). Similarly, the effects of abusive postnatal maternal care on rat BDNF exon IV methylation are observed in the offspring of females that had previously experienced maltreatment, and these effects were only partially eliminated with cross-fostering (Roth et al., 2009). Thus, the developmental effects of early life experiences may have epigenetic consequences that have multigenerational implications.

Research Questions

The goal of this thesis was to expand our current understanding of the mechanisms by which experience of maternal care leads to long-term alterations in gene expression, and ultimately maternal behavior. Towards this aim, the experimental work described in this thesis sought to answer several specific research questions:

Question 1a: What is the time-course whereby postnatal maternal care impacts development of offspring hypothalamic hormone receptor systems?

A missing link in understanding the mechanisms whereby the level of maternal LG experienced leads to variation in hormone receptor and maternal LG levels among offspring is the developmental timing by which these changes in ERα, ERß, and OTR occur within the

MPOA. Developing a time-course of these changes, as well as determining the critical periods of sensitivity to maternal care, would enhance our knowledge of the impact of maternal care on offspring brain development. We hypothesized that estrogen and oxytocin receptor levels would diverge among Low and High LG female offspring during the early postnatal period when LG variation among dams is most pronounced.

Question 1b: Is maternally-induced variation in ERα levels accompanied by variation in DNA methylation and histone protein modifications during postnatal development?

Long-term regulation of gene expression is implied by variation in maternal behavior, ERα mRNA, and DNA methylation among *adult* Low and High LG offspring. Understanding the developmental timing of ERα promoter methylation differences, and whether chromatin modifications might also epigenetically regulate ERα in response to maternal LG, is important for fully understanding the mechanisms that transduce early environmental experiences into long-term changes in gene expression and behavior.

Question 2: Is variation in postnatal maternal care associated with developmental variation in offspring mesolimbic dopamine pathways?

It was previously unknown whether additional pathways contributing to maternal care were also shaped by maternal care. Dopamine systems are critically involved in appetitive maternal behavior, and variation in this system was associated with variation in LG among lactating dams. However, it was unknown whether differences in the level of postnatal maternal care experienced were associated with differences in the mesolimbic dopamine system of offspring, when during development alterations emerged, and what mechanisms might contribute to those changes. Furthermore, exploring whether maternally-induced alterations to the

mesolimbic dopamine system are relevant for other reward-directed behaviors would further enhance our understanding of the development of the mesolimbic dopamine system in response to maternal LG.

Question 3: Does increasing ERα gene expression in the hypothalamus in the early postnatal period impact maternal behavior and/or the mesolimbic dopamine pathway?

Though maternal LG experienced during postnatal development predicts offspring ERα levels in the MPOA and is likely a critical mechanism in driving offspring maternal behavior, the causal relationship between developmentally-induced changes in ERα and maternal behavior had not previously been established. Using developmental over-expression of ERα, we explored the effect of this manipulation on maternal behavior among the offspring of Low and High LG dams, with the hypothesis that ERα over-expression would enhance maternal behavior. This developmental manipulation also allowed us to examine the effect of variation in hypothalamic ERα for the development of the mesolimbic dopamine system. Studies have suggested that estrogen receptor activation influences dopamine neurons at cellular and systems levels, but whether these pathways develop independently, or whether dopamine neurons in the VTA are downstream of MPOA ERα levels during development, was unknown.

Chapter 2: Methods

Animals

Long Evans rats (Charles River Laboratories) were maintained on a 12 hour light-dark schedule (lights on at 0800h) with food and water provided *ad libitum*. Bedding in all cages consisted of soft wood chips, with which animals could build nests, and no nestlets were provided. Pairs of adult virgin females were mated with males in our colony for one week. Females were singly housed 1-2 days prior to parturition. Cages were cleaned on the day of parturition (postnatal day 0 – PN0) and every 7 days until offspring were weaned at PN21; litters were otherwise not manipulated. Pups used for gene expression studies were removed from the litter during cage cleaning in order to minimize stress on the dam and litter; no more than two pups were removed at a time. Weaned offspring were pair-housed by sex. All procedures were performed in accordance with guidelines of the NIH regarding the Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee (IACUC) at Columbia University.

Maternal behavior

Home cage maternal behavior was scored as previously described (Champagne et al., 2003a). Maternal behavior was observed for five 60-minute observation periods daily during postnatal days (PN) 1-6. Observations took place during both the dark (2100h, 0600h) and light phases (1000h, 1300h, 1700h) of the circadian cycle. Behavioral observations were made every 3

minutes during each observation period for a total of 600 observations per litter. Behaviors scored included contact with pups, nursing posture, pup licking/grooming, nest-building, eating, drinking, and self-grooming. Frequency of a behavior was calculated as the number of observations of the behavior divided by the total number of observations. Licking/grooming (LG) behavior is normally distributed over a large number of dams (Champagne et al., 2003a). Low and High LG dams were selected from cohorts of 40 dams and defined as engaging in LG frequencies that were either one standard deviation below (Low LG) or above (High LG) the mean LG of the cohort. In one cohort, Low (n=4) and High (n=4) LG dams were observed on PN10 for one hour and the number and duration of LG bouts (discrete, continuous periods of LG) were continuously recorded (see Franks et al., 2011 for detailed methodological description).

Tissue collection

Female offspring of Low and High LG dams were sacrificed by rapid decapitation at the day of birth (PN0), PN6, PN21 at weaning, as young adults at PN66, or as characterized dams on postpartum day 6 (n=6-8 animals/ group). Dissected brain tissue samples for gene expression and epigenetic analysis were from female siblings sampled at multiple developmental time points (*i.e.,* one female per litter per time point). Whole brains were removed and immediately snap-frozen and stored at -80°C until further processing. Three brain regions were carefully dissected from one half of each brain in a cryostat cooled to -20°C and processed for RNA/DNA extraction: 1) the anterior ventral medial region of the hypothalamus (Bregma 0.12 to -0.72), containing the MPOA; 2) the ventral midbrain containing the VTA and substantia nigra (Bregma -4.68 to -6.48); and 3) the ventral striatum containing the nucleus accumbens core and shell

(Bregma 2.28 to 0.72). The MPOA of the other half of each brain was dissected and used for chromatin immunoprecipitation (ChIP) assay.

RNA and DNA extraction

Dissected brain tissue samples were weighed and then homogenized in 700µl lysis buffer RLTplus (Qiagen) with 1% beta mercaptoethanol using a tissue homogenizer (Omni) for 15 seconds. RNA and DNA were extracted using a dual RNA-DNA extraction kit (Qiagen). cDNA was created from RNA using a reverse transcription kit (Applied Biosystems). Samples were stored at -20°C until further processing.

Gene expression

Relative gene expression was measured by real-time semi-quantitative PCR on a 96-well 7500Fast qPCR machine (Applied Biosystems) using SybrFast. All primers were designed to span or be located on separate exons and had 87-110% efficiency in a standard curve, with single melt peaks. Primer pairs are included in **Table M1**. Calculations of relative gene expression were conducted using the 2-ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) with cyclophilin-A and beta-actin as control genes and normalized to PN0 Low LG offspring.

Table 2.1: Real-time qPCR primers

Gene name	Gene ID	Forward primer	Reverse primer
BDNF - total	NM_012513	CCATAAGGACGCGGACTTGTAC	AGACATGTTTGCGGCATCCAGG
Beta-Actin	NM_031144	ATGGATGACGATATCGCTGCG	GGTGACAATGCCGTGTTCAAT
Cdkn1c	NM_001033757.1	ACGTCTGCGATGAGTTAGTTTAGA	AAGGTCCCAGCCGAAGCCCA
Cyclophilin A	NM_017101	ATGGTCAACCCCACCGTGTTCTTC	ATCCTTTCTCCCCAGTGCTCAGAG
DAT	NM_012694.2	TCGCCACACTCCCGCTGTCT	TGGAACTCATCGACGAGCCCAGT
Dnmt1	NM_053354.3	GTGGGATGGCTTCTTCAGTA	GGCTTGGTCACAAAACAAAC
Dnmt3a	NM_001003958.1	GGGGCCCCAGCTGAAGGAGA	GCCCCGGGAGCCCTCCATTT
DRD1a	NM_012546	GGCTGCCAGCGGAGAGGGAT	GACGGCCGCACAGACAAGGG
DRD2	NM_012547	ACACCAAGCGCAGCAGTCGA	GCGGGCAGCATCCATTCTCCG
DRD3	NM_017140	GGCTGCATCCCATTCGGCAGT	GGGGGCTGCAGGTGTGACAA
ER-alpha	NM_012689.1	GCCTTCTACAGGTCCAATTCTGAC	ACAGCACAGTAGCGAGTCTCC
ER-beta	NM_012754.1	GCAGAACCTCAAAAGAGTCCTTGG	ACGCCGTAATGATACCCAGATG
Lmx1b	XM_001069713.2	GCTCAAGGAGGGCCAGCTGC	CCTGCCCCTTGGCTGGCTTC
Nurr1 /a	U72345.1	TAAAAGGCCGGAGAGGTCGTC	CTCTCTTGGGTTCCTTGAGCC
OTR	NM_012871	TTCTTCGTGCAGATGTGGAG	GAGCATGTAGATCCACGGGT
Pitx3	NM_019247.1	CGGGACACACTAGCCCTCCCT	TGAGGCCGAGGCCTTCTCCG
TrkB-FL	NM_012731	GATCTTCACCTACGGCAAGC	TCGCCAAGTTCTGAAGGAGT

Immunohistochemistry

At PN6, female offspring (n=5-6 per group) were rapidly decapitated and whole brains placed into 4% paraformaldehyde in PBS for 48-hour fixing (2 hours at room temperature and then at 4°C). Adult females were swabbed for estrous state (Low LG females: estrous & proestrous n=2, diestrous n=3; High LG females: estrous & proestrous n=5, diestrous n=2) prior to being anesthetized with a terminal dose of ketamine/xylazine and transcardially perfused with

PBS followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde at 4°C. Brains were then cryoprotected in 30% sucrose in PBS at 4°C until isotonic, and stored at -80°C until further processing. 40μm coronal sections were sliced on a cryostat and floated into PBS. Sections were washed in PBS and blocked with normal goat or donkey serum before incubating in primary antibody in PBS with Triton-X (hypothalamic sections: rabbit-anti-ERα, 1:3,000, Santa Cruz Biotechnology; midbrain sections: rabbit-anti-tyrosine hydroxylase, 1:10,000, Santa Cruz Biotechnology) at 4°C overnight. Sections were then washed in PBS, incubated in secondary antiserum [PN6 females (MPOA) and adult females (MPOA and VTA): biotinylated goat-anti-rabbit (Vectastain), followed by chromogen visualization (Vector SG); PN6 females (VTA): Cy2 conjugated donkey-anti-rabbit, 1:2000, Santa Cruz Biotechnology] for 1hour at room temperature. After washing, sections were dehydrated in a series of ethanol washes, cleared with xylene, and coverslipped with DPX. Immunofluorescent slides were stored in a light-proof box at 4°C before and after imaging. Brains of juveniles used to examine effects of *Esr1* over-expression were processed similarly with the following exceptions. Animals were perfused and brains fixed on the day that juveniles completed maternal sensitization testing. In one series of staining, all sections were dual labeled for GFP (green fluorescent protein) and total ERα (primary antibodies: chicken-anti-GFP, 1:1000, Aves Labs; rabbit-anti-ERα, 1:3000, Santa Cruz Biotechnology) using immunofluorescent secondary antibodies (Alexa Fluor 488 goat-anti-chicken and Alexa Fluor 546 donkey-anti-rabbit, 1:1000, Life Technologies). Sections were washed, mounted on gelatin-coated slides, and cover-slipped with ProLong Gold Antifade Reagent with DAPI (Life Technologies). In a second series of staining, identical sections were stained specifically for human ESR1 protein (primary antibody: mouse-anti-hESR1, 1:3000, Life Technologies product

49-1002; incubated over-night at 4°C; secondary antibody: biotinylated rabbit-anti-mouse, followed by chromogen visualization as described). Slices were washed, mounted on gelatin-coated slides, dehydrated in a series of ethanol washes, cleared with xylene, and cover-slipped with DPX. For clarity, total estrogen receptor-alpha will be referred to as "ERα" while virally over-expressed human estrogen receptor-alpha will be referred to as "ESR1." In a third series of staining, 8-11 sections per animal that including the ventral midbrain were immunofluorescently stained for TH as previously described and cover-slipped with ProLong Gold Antifade.

Imaging and cell count

Slides were imaged on an Olympus light microscope fitted with fluorescent filters when appropriate. Aperture adjustments were made as deemed necessary to maintain similar levels of fluorescent intensity between samples. Notations were kept for each image as to the relative anterior-posterior position of the section. Cell counts were determined using MCID Core software (InterFocus Imaging Ltd, UK) programmed for approximate cell size and an acceptable range of stain intensity. An observer blind to condition outlined each region of interest for nuclei-specific analysis. 4-10 sections along the A-P axis were analyzed per brain region per animal. Region area and density of immunoreactive cells were also recorded. An example of the region considered the MPOA is given in **Figure 2.1**. Within the ventral midbrain, nuclei-specific analysis was performed and included the following regions, as determined by *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 2005; see **Figure 2.2**): rostral VTA (VTAR; parafasciculus retroflexus area by Ikemoto 2007), parabrachial pigmented nucleus of the VTA (PBP), paranigral nucleus of the VTA (PN), parainterfascicular nucleus of the VTA (PIF), rostral linear nucleus of the raphe (RLi), caudal linear nucleus of the raphe (CLi), interfascicular

nucleus (IF), and the substantia nigra pars compacta dorsal tier (SNCD), medial tier (SNCM), ventral tier (SNCV), substantia nigra pars lateralis (SNL), and substantia nigra pars reticulata (SNR). Statistics were performed on an animal's mean total cell count, except where noted.

Figure 2.1 Medial preoptic area of the hypothalamus

A schematic representing the region of MPOA counted for ERα-expressing cells. This example is at the level of Bregma -0.24 mm; Paxinos & Watson, 2005.

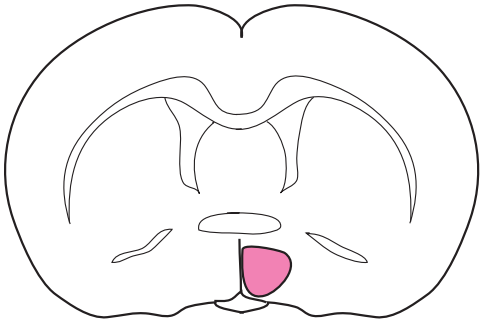
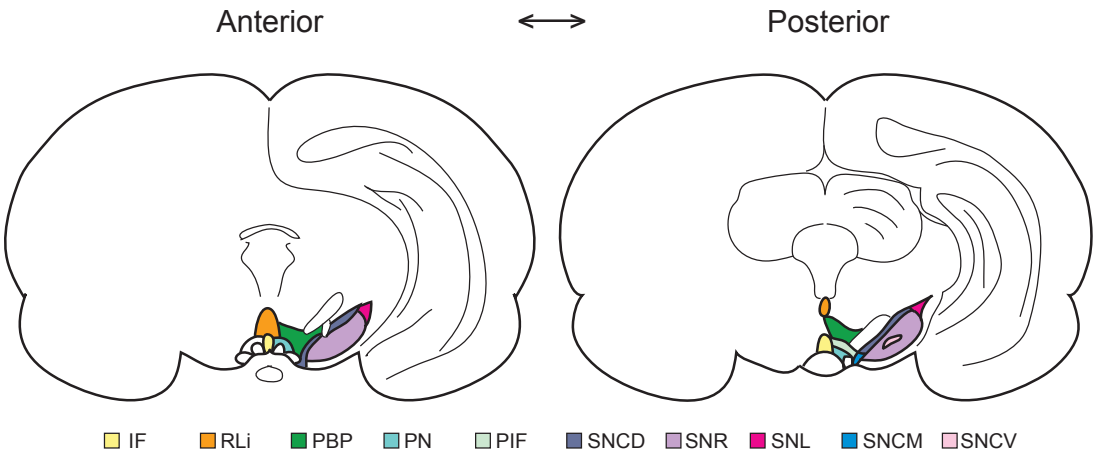


Figure 2.2 Nuclei of the ventral tegmental area and substantia nigra

A schematic representing VTA and SN nuclei counted for tyrosine hydroxylase-expressing cells. On the left is an example from the anterior ventral midbrain (Bregma -5.28 mm), and on the right is a more posterior (Bregma -6.24 mm) example including each of the regions taken into account.



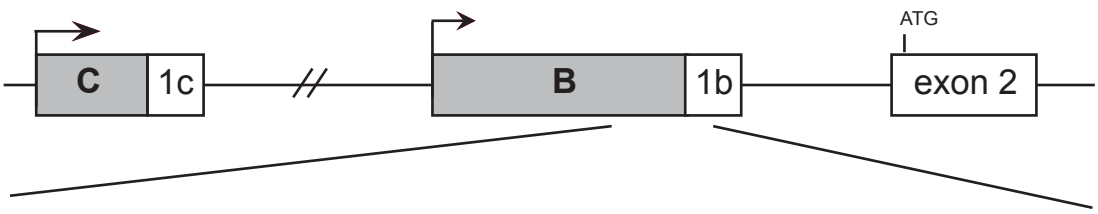
Bisulfite conversion and pyrosequencing: MPOA

CpG Methylation analysis was conducted using DNA extracted from the MPOA of female offspring of Low and High LG dams, and from Low and High LG dams on postpartum day 6 (n=3-9 animals/group/timepoint). 1µg of DNA purified from the MPOA was bisulfite-converted and the *Esr1* regulatory region "B/1b" (GenBank X98236.1; named as in Freyschuss & Grandien, 1996; see **Figure 2.3**) was amplified with a biotinylated reverse primer (EpigenDX). Transcription initiated from promoter B includes a 5' untranslated exon "1b" (Freyschuss and Grandien, 1996), and this B/1b sequence includes the same 14 CpG sites assessed by bisulfite sequencing in Champagne *et al.* (2006.) Samples were then pyrosequenced on a 96-well pyrosequencer (PSQ™96HS; Qiagen) with two sets of sequencing primers (EpigenDX: ADS1511, ADS1512). Percent methylation was determined by the ratio of C to T incorporation at 14 CG sites in the sequence. Only samples that passed internal positive control standards were included in the analysis.

Figure 2.3 Estrogen receptor-alpha regulatory region

Schematic of the *Esr1* gene regulatory region, including the proximal B promoter and 5'UTR 1b; the first translated exon is Exon 2 (adapted from Freyschuss & Grandien, 1996; Ensembl ENSRNOT00000026350). 14 CpG sites in the B/1b regulatory region were analyzed by bisulfite pyrosequencing and are numbered and indicated in bold.

Figure 2.3 Estrogen Receptor-α regulatory region



CG[1]gactc**CG**[2]gctgccattcattcag**CG**[3]tcctgcagaagcccagctgc**CG**[4]**CG**[5]tctgc**CG**[6]gg
aggggctgccaagtgccctgcctactggctgcttcc**CG**[7]agagtccctgccactccacatacaaacaca
tccacaca**CG**[8]ctctgccttgatcacacac**CG**[9]**CG**[10]ccact**CG**[11]atcatt**CG**[12]agcacattccttcc
ttc**CG**[13]tcttactgtctcagctcttgacttctacaaacccatggaacatttctggaaaga**CG**[14]

Bisulfite conversion and pyrosequencing: ventral midbrain

CpG methylation analysis was conducted using DNA extracted from the ventral midbrain (n=6-8 animals/ group). 1ug of DNA purified from the VTA was bisulfite-converted and cleaned using EpiTect Bisulfite Kit according to manufacturer's instructions (Qiagen). The tyrosine hydroxylase (*Th*) promoter region was amplified using Pyromark PCR (Sigma) with a biotinylated reverse primer. This regulatory region contained 10 CpG sites (-269 to -94) and has been implicated in epigenetic regulation of *Th* in culture (He et al., 2011). A portion of the bisulfite-converted DNA from each sample was run on a gel to ensure specificity. Samples were then pyrosequenced on a 24-well pyrosequencer (Qiagen) with two sets of sequencing primers (see **Table 2**). All pyrosequencing primers were designed using PyroMark software (Qiagen). Percent methylation was determined by the ratio of C to T at CpG sites in the sequence. Only samples that passed internal positive control standards were included in the analysis.

Table 2.2: ChIP qPCR primers

Gene name	ID	Forward primer	Reverse primer
ER-alpha 1b promoter 5'UTR	X98236	CACACACCGCGCCACTCGAT	ACACCGATCCTACCCTGCTGGT
ER-alpha 1b promoter upstream	X98236	AGATGGGCGCTGGAACCGGAG	TCTGCTGTTGGCTATGTGGCTTGC
ER-alpha exon1	X98236	CCAGGTGGCTCATCCGCTGC	TTGAACACGGCGGGCTTGCT

Chromatin immunoprecipitation (ChIP)

DNA and protein from microdissected MPOA samples (n=6-7 animals/group) were cross-linked in 1ml cold PBS with 1% formaldehyde and protease inhibitors for 15 minutes at

room temperature, with rotation. Tissue was washed in PBS and protease inhibitors 3 times, and then homogenized in 900µl PBS and protease inhibitors with a tissue homogenizer (Omni) for 10 seconds. Samples were spun down at 2000rpm for 5 minutes at 4°C and resuspended in ice cold Cell Lysis Buffer (Millipore) with protease inhibitors, and incubated on ice for 15 minutes with occasional gentle vortexing. Samples were spun down again and resuspended in Nuclear Lysis Buffer (Millipore) with protease inhibitors. Sonication was done in an ice bath using a Bioruptor (Diagenode) for 30 cycles of 30 seconds on, 30 seconds off. Temperatures were kept at 4-6°C. Gel analysis of sheared DNA confirmed DNA fragments between 200-700bp. Samples were diluted 10-fold in ChIP Dilution Buffer (Millipore) with protease inhibitors: 20µl from each was saved as "Input," and 50µl was used for immunoprecipitation with antibody [anti-trimethyl-Histone H3 lys 4 (H3K4me3) or anti-trimethyl-Histone H3 lys9 (H3K9me3): ChIP grade from Upstate/Millipore]. Immunoprecipitation, washing, de-crosslinking, clean-up, and elution was conducted with Magna ChIP kit (Millipore). Elutant was stored at -20°C until analysis with qPCR. Input (5µl) of samples was de-crosslinked by incubation with protinase K at 62°C for >4 hours followed by column-based purification (Qiagen PCR cleanup kit). Samples and input were analyzed by qPCR and "% Input" calculated. Primers used in qPCR (see **Table 2.2**) were designed to amplify several genomic regions including an upstream *Esr1* promoter region, the *Esr1* B/1b regulatory region corresponding to the sequence analyzed by bisulfite pyrosequencing, and first translated *Esr1* exon (Ensembl ENSRNOT00000026350 exon 2).

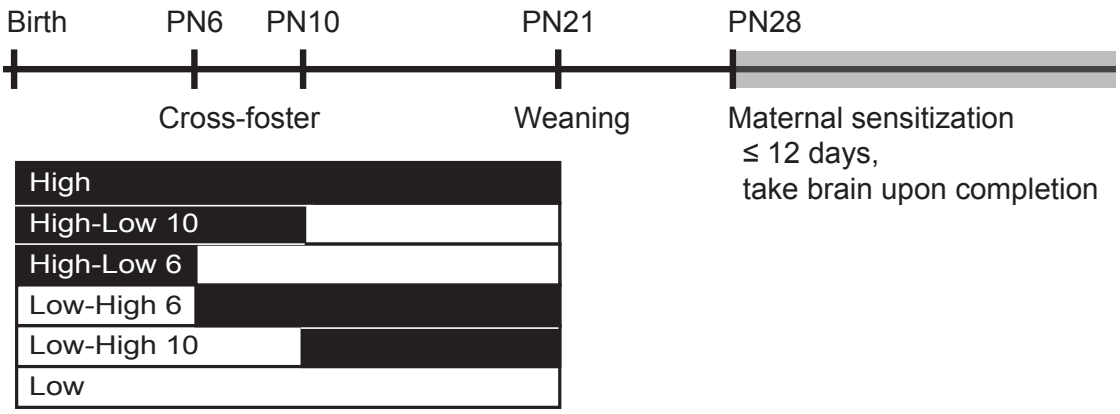
Cross-fostering

In order to assess sensitive periods for the effect of maternal care on offspring gene expression and behavior, additional cohorts of dams were mated. Offspring of Low LG and

High LG litters were divided into three conditions: 1) reared by birth dam from birth until weaning, 2) cross-fostered at PN6 between Low LG and High LG dams ("Low-High 6" and "High-Low 6"), or 3) cross-fostered at PN10 between Low LG and High LG dams ("Low-High 10" and "High-Low 10"; see **Figure 2.4**). Each Low LG litter was paired to a High LG litter and offspring were switched between them on cross-fostering days. At PN6 one female and two male offspring were cross-fostered together between Low and High LG litters. At PN10, one female and one male sibling were cross-fostered together between Low and High LG litters. Pups were cross-fostered between litters born on the same day to avoid differential suckling or nest competition by cross-fostered *vs.* biological offspring; pups were monitored after fostering and all pups were successfully fostered. Pictures of the unique back pattern of each pup were taken to identify cross-fostered offspring. Offspring were singly housing at weaning in preparation for maternal sensitization testing.

Figure 2.4 Timeline of cross-fostering and maternal sensitization groups

Offspring were cross-fostered between Low LG and High LG dams at PN6, PN10, or were left with their initial dams as non-fostered controls. All animals were weaned at PN21 into single housing and maternal sensitization testing began at PN28. Six groups were created by cross-fostering: control Low, control High, cross-fostered at PN6 ("Low-High 6" and "High-Low 6"), and cross-fostered at PN10 ("Low-High 10" and "High-Low 10").



Juvenile maternal sensitization

Juvenile (<PN40) virgin female offspring of Low and High LG dams were tested for maternal sensitization in two experiments (see **Figure 2.4** and **Figure 2.5**). In addition, female offspring cross-fostered between Low LG and High LG dams at PN6 (n=12 animals/ group) and PN10 (n=7-8 animals/ group) were assessed in this test in order to characterize the sensitive period for the development of this behavior. Maternal sensitization involves exposing test subjects to neonates daily until they display maternal behavior toward the neonates; continual pup exposure or treatment with hormones are known to induce maternal responsivity in otherwise non-maternal animals including virgins (Rosenblatt, 1967; Fleming and Rosenblatt, 1974; Mayer et al., 1979; Fleming, 1986). Maternal sensitization was conducted as previously described (Bridges and Ronsheim, 1990; Champagne et al., 2001). Animals were tested daily between 1500 and 1700 beginning on PN28. Each day, three recently fed, 2-5 day-old pups from a donor litter were placed in three quadrants in the cage (one pup per quadrant) away from the nest site. The test animals were observed continuously for 1 hour for pup retrieval, crouching over the pups in a nursing position, and pup licking/grooming. Donor pups were left in the cage for 23 hours. On test days 2–12, pups from the previous session were removed, and 30 minutes later a new set of recently fed pups of the same age were introduced into each test cage, thereby commencing another 1-hour test session. Testing continued for 12 consecutive days or until a female displayed full maternal behavior, whichever occurred first. Females were scored as fully maternal if they retrieved all three test pups to the nest, grouped them in the nest, and crouched over them within the 1-hour test session. Juveniles failing to respond in this way within the 12-day test period were assigned a score of 13 so as to discriminate from animals that engaged in

two-consecutive days of full behavior on day 12. After induction of maternal behavior or on day 12, juveniles were sacrificed and brains snap frozen for analysis of gene expression.

Conditioned place preference (CPP) behavior testing

In order to evaluate preference for naturally rewarding stimuli, animals were tested for conditioned place preference for a target stimulus and a control stimulus. Testing took place over 8-9 consecutive days, which included habituation, conditioning, and preference testing. Testing began on PN24 so that all animals would complete prior to the onset of puberty.

Apparatus

The CPP apparatus used was a 3-chamber Plexiglass apparatus with three equal-sized (20 x 20cm) chambers separated by small openings. The outside two chambers were decorated with distinct contextual cues that the animals could distinguish and the center chamber had plain black walls. Fresh bedding was placed on the floor of each chamber, and the chambers were thoroughly cleaned between animals.

Habituation and exploration

In order to allow animals to habituate to the CPP chamber and to identify any preexisting chamber preferences, animals were allowed to freely explore the entire apparatus for 30 minutes on Day 1 of testing. Movements were recorded using ANY-maze (Stoelting, Wood Dale, IL) and initial chamber preference was assessed (defined as spending > 50% difference in time spent between the two outside chambers). In cases where there was a preference, the control stimulus was placed in the preferred chamber. In the absence of preference, the stimuli were randomly placed in either chamber.

Conditioning for 1) high fat diet or chow, or 2) sibling or familiar toy

In order to evaluate whether the frequency of maternal LG experienced during infancy predicted offspring preference for natural rewards during the juvenile period, CPP for two different pairs of stimuli was assessed; 1) high fat diet (HDF; reward) *vs.* chow (control) (**Table 2.3A)**, or 2) a sibling/cagemate (reward) *vs.* a familiar toy (control) (**Table 2.3B)**. Conditioning took place over 6 days, during which female offspring were conditioned to associate one side of the chamber with one of the two stimuli and the other side of the chamber with the control stimulus. CPP testing for each stimulus pair were conducted in separate cohorts of offspring (i.e. offspring not previously tested for CPP). HFD has been previously shown to be naturally rewarding for rodents with no training necessary to induce exploration and consumption (Teegarden & Bale, 2007). To ensure animals would explore and eat the novel HFD, all animals were exposed to HFD (D12492; Research Diets) in their home cage 2 days prior to habituation. Two pellets were introduced to the home-cage and checked 4 hours later to examine consumption. All animals consumed some if not all of the HFD pellets. Four pellets of HFD or chow were placed on the bottom of the assigned chamber during each conditioning session. In the sibling/cagemate conditioning, the social partner was a sibling pair-housed with the test subject. The familiar toy (control) was a hollow plastic tube that had been present in the home-cage. During conditioning, the animal was restricted to one side of the chamber (containing the stimulus) for 60 minutes. The stimulus that was presented first during training was counter-balanced (see **Table 2.3**).

Table 2.3 Conditioned place preference test design

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
A	Habituation Trial 30min	Conditioning (HFD) 60min	Conditioning (chow) 60min	Conditioning (HFD) 60min	Conditioning (chow) 60min	Conditioning (HFD) 60min	Conditioning (chow) 60min	Preference Test 60min	
	Habituation Trial 30min	Conditioning (chow) 60min	Conditioning (HFD) 60min	Conditioning (chow) 60min	Conditioning (HFD) 60min	Conditioning (chow) 60min	Conditioning (HFD) 60min	Preference Test 60min	
B	Habituation Trial 30min	Conditioning (sibling) 60min	Conditioning (familiar toy) 60min	Conditioning (sibling) 60min	Conditioning (familiar toy) 60min	Conditioning (sibling) 60min	Conditioning (familiar toy) 60min	Preference Test 60min	Preference Test: vehicle or Haloperidol 60min
	Habituation Trial 30min	Conditioning (familiar toy) 60min	Conditioning (sibling) 60min	Conditioning (familiar toy) 60min	Conditioning (sibling) 60min	Conditioning (familiar toy) 60min	Conditioning (sibling) 60min	Preference Test 60min	Preference Test: vehicle or Haloperidol 60min

Preference test

On the 8th day of chamber exposure, animals were tested for chamber preference. During the preference test (1 hour in duration), animals were permitted to explore the entire apparatus in the absence of any stimuli, and movements were recorded with ANY-maze. Based on the chamber exploration during testing, a preference score was calculated as time spent in the reward-associated chamber divided by the time spent in both outer chambers together: Preference score = $(T_{HFD}) / (T_{HFD} + T_{chow})$ or Preference score = $(T_{sibling}) / (T_{sibling} + T_{toy})$. In order to evaluate whether D2-like receptors mediated social preference, chamber preference was evaluated on the 9th day, following an injection of either DMSO or the dopamine receptor (D2 preferring) antagonist Haloperidol (0.3 mg/kg i.p., Sigma; based on Giordano et al., 1990; Zhao and Li, 2009), 20 minutes prior to testing.

Adenovirus

Pre-packaged human type-5 adenoviruses containing human ESR1 (Ad-ESR1, SL100776; NM_000125) or GFP (Ad-GFP, SL100708) under the control of a constitutively active CMV promoter were purchased from SignaGen Laboratories (Rockville, MD). Both viruses lacked early phase genes E1 and E3 rendering them replication deficient, and had titers of $1x10^{10}$~$1x10^{11}$ PFU/ml. Adenovirus was chosen over other virus types (eg AAV, lentivirus) primarily because it is able to begin expressing quickly after introduction to a host (within as little as two days; Penuelas et al., 2005; Hu et al., 2010), and an important aspect of the current study was to understand whether elevated developmental expression was related to behavior. Adenovirus is nearly 100% transduction-efficient in dividing and non-dividing cultures, does not

cross synapses, is fairly non-oncogenic, and has been found to have lasting expression in vivo for at least 9 months (Rahim et al., 2011). Although expression is transient because adenoviruses do not integrate into the host, pilot testing revealed that injection into neonatal brain could be detected at 6 weeks after injection. This time course of expression was within the planned time course of behavioral testing. All virus handling and injections were performed in a biosafety cabinet and approved by Columbia University Environmental Health and Safety and IACUC.

Adenovirus Injections

Neonatal female pups received stereotaxic injections of either Ad-GFP or Ad-ESR1 on PN 2 or PN3 (see **Figure 2.5**). In order to test the hypothesis that higher MPOA ERα expression beginning early in postnatal development leads to increased levels of maternal behavior, the injections needed to occur as early as possible after birth. However, to maximize survival probability and predictability of Low and High LG litters, injections were not performed on PN1. Pups selected for injection were cryoanesthatized in a plastic container on wet ice for 15 minutes, until nonresponsive to foot pinch (Auger et al., 2002; Bishop et al., 2005; Davidson et al., 2010). Two 10μl Hamilton syringes were used to deliver 0.4μl microinjections of virus per animal. Each needle was marked and dedicated to one virus over the course of the study. The needles were fit into a MicroSyringe Pump Controller (World Precision Instruments) and attached to a stereotax adapted for neonatal rats with palate bar and rubber ear cuffs. Bregma is visible through the skin under bright light through PN3 and was used to guide needle placement. The skull of neonatal rats (≤PN3) is thin and easily pierced by a needle and thus no incisions were needed (Marquis et al., 2006; Davidson et al., 2010). In order for virus to be incorporated in the MPOA without bilateral injections that increase the risk for error, injections of Ad-GFP or Ad-

ESR1 were aimed at the ventral 3rd ventricle. (Pilot testing with Ad-GFP revealed staining within the MPOA in addition to the ventricle walls.) The needle was therefore centered at Bregma and lowered to -5mm over the course of 30-40 seconds. Virus was infused via the MicroSyringe Pump Controller at a rate of 4nl/second to ensure absorption of the fluid into the brain without increasing internal pressure. After a rest period of 60 seconds to prevent backfill, the needle was slowly removed over the course of 30 seconds, at which point the animal was transferred to a warming pad to recover. Liquid topical adhesive was applied to the point of injection to minimize potential for infection. This had the added benefit of preventing some hair growth on the head for later identification. Additionally, as young as PN2, Long Evans rat back-patterns become faintly visible, and thus photographs of each pup's unique back-pattern were taken after injection in order to identify the pups at later stages. Pups typically recovered and were mobile within 10 minutes, and were away from their home cage for approximately 30 minutes. When all siblings from a litter recovered, they were returned to their dam together. 100% of injected pups survived. Needles and equipment were cleaned with 70% ethanol after each injection, and with fresh 10% bleach after each day of injections.

ESR1 over-expression cohort composition

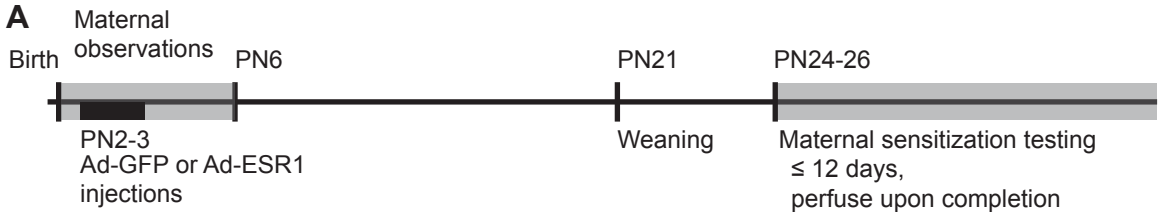
Maternal LG scores from the first two postnatal days were used to predict which litters would be Low LG or High LG and pups from these initial Low and High LG litters were selected for injection. Up to four females per litter were injected: two with Ad-GFP and two with Ad-ESR1. Of 40 dams bred, 35 gave birth resulting in 20 litters selected for injection as potential Low or High LG, and a total of 69 pups were injected with virus. Of the 20 litters, analysis of the full week of maternal observations revealed 4 litters to be Low LG and 5 to be High LG.

Two female offspring from each of two additional Low LG litters that did not receive injections were also included as controls. Based on the number of donor pups available for maternal sensitization testing, 48 offspring were tested, as per **Figure 2.5**.

Figure 2.5. Over-expression study design

(A) Maternal observations took place from PN1-6. Pups were injected with either Ad-GFP or Ad-ESR1 on PN2 or PN3. Maternal sensitization testing began between PN24-26 and animals were perfused upon completion or after 12 days of testing, whichever came first. (B) The cohort composition of the 48 control and *Esr1* over-expressing females tested for maternal sensitization latency.

Figure 2.5



B

Females tested	Low	Mid	High
Control/ Ad-GFP	11	4	10
Ad-ESR1	8	9	10

Statistics

All statistics were performed using SPSS (PASWS, IBM, version 18.0). Litters were not standardized for size or male/female ratio and these variables were used as covariates in analyses. Two-tailed student's t-test was used for single-comparisons among High and Low LG animals. Main effects were determined using MANOVA with independent and dependent variables as noted within the results. Repeated measures ANOVA was used to determine effects

with multiple time-points sampled (behavior and gene expression) where described within the results. Generalized Wilcoxon test was used for Kaplan-Meier survival analysis, as indicated. Outliers were defined as more than 2 standard-deviations away from the group mean and were removed from analysis when stated in order to avoid type-II error. All significance thresholds were set at $p<0.05$.

Chapter 3: Developmental timing of the effects of maternal care on gene expression and epigenetic regulation of hypothalamic hormone receptor levels

Introduction

Hypothalamic hormone receptor and neuropeptide levels have been implicated in the expression of parental behaviors (Pedersen et al., 1982; Fahrbach et al., 1984; Fahrbach et al., 1985; Francis et al., 2000; Champagne et al., 2001; Francis et al., 2002; Champagne et al., 2003b; Champagne et al., 2006; Feldman et al., 2010) and particularly with the onset and maintenance of maternal behavior. Within the hypothalamus, the medial preoptic area (MPOA) is critical for maternal behavior (Kalinichev et al., 2000a; Numan and Insel, 2003; Febo, 2005; Numan, 2006); Numan & Insel, 2003; Numan 2006; Kalinichev et al 2000), and studies in rodents have demonstrated that activation of oxytocin receptors (OTR) and estrogen receptor-alpha (ERα) in this brain region are necessary for maternal behaviors including nest building, pup retrieval, and licking/ grooming (LG) (Pedersen et al., 1982; Fahrbach and Pfaff, 1986; Ahdieh et al., 1987; Pedersen et al., 1994; Bale and Dorsa, 1995, 1997; Ogawa et al., 1998; Young et al., 1998; Champagne et al., 2001; Meddle et al., 2007; Ribeiro et al., 2012; Spiteri et al., 2012).

Variation in ERα levels critically contribute to individual variation in maternal behavior, and appear to mediate variation in OTR binding (Fahrbach et al., 1984; Ahdieh et al., 1987;

Champagne et al., 2001; Champagne et al., 2003b; Champagne et al., 2006). Rat dams that engage in high frequencies of pup LG (High LG) have higher levels of OTR binding and ERα mRNA in the MPOA compared to Low LG dams (Champagne et al., 2001; Champagne et al., 2003b). There is evidence that the early maternal environment contributes to variation in offspring hormone receptor levels and maternal behavior in adulthood (Francis et al., 1999; Champagne et al., 2001; Champagne et al., 2003b; Champagne et al., 2006; Ahern and Young, 2009; Lukas et al., 2011). Female offspring of High LG dams exhibit increased LG of pups in comparison to offspring of Low LG dams (Francis et al., 1999; Champagne et al., 2001; Champagne et al., 2003a) and offspring hormone receptor variation is associated with the level of maternal LG in rats, such that females reared by High LG dams have elevated levels of OTR binding and ERα mRNA in the MPOA compared to Low LG offspring. Moreover, cross-fostering studies (where fostering is conducted on the day of birth) suggest that it is the experience of LG during the postnatal period that shapes these outcomes.

However, little was known about the developmental timing of these individual differences in receptor levels and behavior in response to maternal LG. We hypothesized that differences in hormone receptor levels among female offspring of Low and High LG dams would emerge in the early postnatal period when differences in maternal LG are most pronounced. The experiments described in this chapter examined ERα, ERß, and OTR across development in the MPOA of females reared by High and Low LG dams. To determine whether epigenetic mechanisms contributed to differences in ERα (*Esr1*) during development, both DNA methylation and chromatin modifications associated with the *Esr1* regulatory region were analyzed. Finally, in order to assess sensitive periods for the effect of maternal LG on offspring brain development and behavior, female offspring were cross-fostered between Low LG and

High LG dams at two different postnatal time points and tested for maternal sensitization behavior and hormone receptor gene expression.

Results

Comparison of postpartum maternal LG behavior in Low LG vs. High LG dams

Overall LG frequency (averaged across PN1-6) was significantly elevated among High compared to Low LG dams [representative cohort (9 Low, 7 High LG dams) with litters used for gene expression and ChIP analysis: $t(1,14)=8.23$, $p<0.001$; **Figure 3.1A**] and repeated measures analysis with postpartum day as a within-subject factor and maternal LG as a between-subject factor indicated a significant effect of day [$F(5,65)=6.54$, $p<0.001$] and a significant effect of maternal LG [$F(1,13)=50.74$, $p<0.001$], but not a significant interaction between the two ($p>0.21$). LG decreased across the postpartum period in both High and Low LG dams and group differences in LG were apparent across the postpartum period (with High and Low LG dams becoming more similar in frequency of LG on PN6; **Figure 3.1B**). Additional observations were conducted in one cohort of dams on PN10 in order to examine the number and length of LG bouts among Low and High LG dams at this later time point. No differences were found among Low and High LG dams in the number of LG bouts on PN10 ($p>0.2$; **Figure 3.1C**), however, the average LG bout length was found to be significantly longer in duration in High LG compared to Low LG dams [$t(1,6)=2.70$, $p<0.05$; **Figure 3.1D**]. This analysis suggests that behavioral differences between High LG and Low LG dams extend beyond PN6.

Figure 3.1

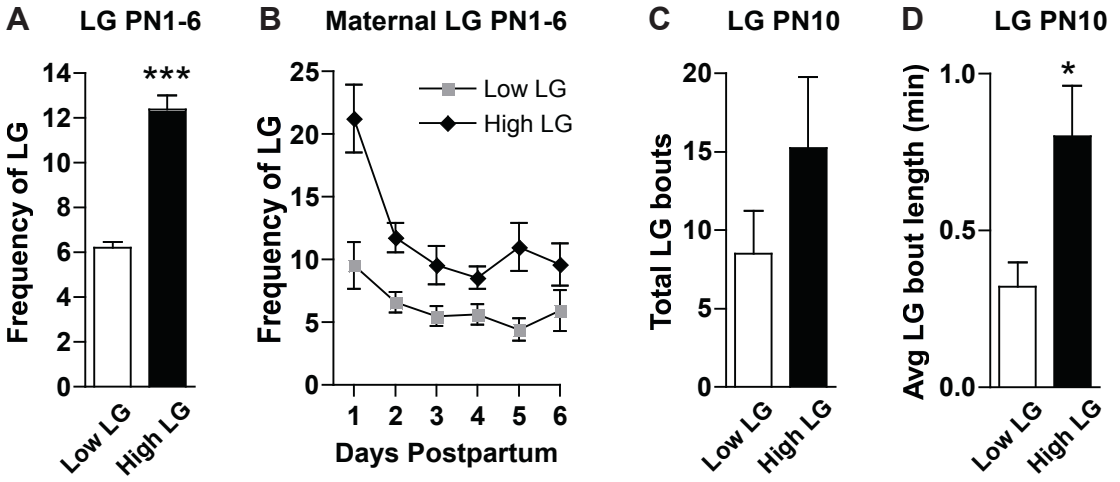


Figure 3.1 Maternal licking/grooming levels

Mean ± SEM LG frequency (% of observations) for Low LG and High LG dams (A) averaged across the first 6 days postpartum, (B) on each of the first 6 days postpartum. Mean ± SEM LG bouts (C) and average LG bout duration (D) on postpartum day 10 by Low LG and High LG dams. *$p<0.05$, ***$p<0.001$.

Developmental timing of the effects of LG on gene expression in the MPOA

To determine whether neuroendocrine changes apparent in adult female offspring that experience varying levels of maternal care (Champagne et al., 2003b; Champagne et al., 2006) can be observed at the level of transcription during postnatal development, brains were collected from female offspring of Low LG and High LG dams at postnatal days 0, 6, 21, and 66 (**Figure 3.2A**). Using semi-quantitative PCR, levels of ERα, ERß, and OTR mRNA were analyzed in the MPOA at each developmental age. Two-way ANOVA (with maternal LG and age as factors) indicated a main effect of maternal LG [$F(1,54)=7.69$, $p<0.01$] and age [$F(3,54)=83.79$, $p<0.001$], as well as a significant maternal LG by age interaction [$F(3,54)=3.41$, $p<0.05$] on relative ERα mRNA levels (**Figure 3.2B**). Subsequent analysis indicated ERα mRNA levels

were significantly elevated among High LG female offspring compared to Low LG offspring at PN21 ($p<0.01$) and in adulthood ($p<0.05$), whereas no group differences were found at birth or PN6 ($p>0.13$). Three animals were determined to be outliers in ERß mRNA expression and were removed from analysis (one PN21 Low, one PN66 Low, and one PN66 High LG sample). ERß mRNA levels varied as a function of maternal LG and age [maternal LG: $F(1,51)=13.28$, $p<0.001$; age: $F(3,51)=54.59$, $p<0.001$], with a significant interaction between maternal LG and age [$F(3,51)=5.61$, $p<0.01$; **Figure 3.2C**] on the expression of this isoform of the ER receptor. Similar to ERα, ERß mRNA levels were elevated among High LG female offspring compared to Low LG offspring at PN21 ($p<0.01$) and in adulthood ($p<0.05$) while no differences were found at birth or PN6 ($p>0.45$). There were trends for an effect of litter size [$F(7,54)=2.01$, $p=0.07$] and male/female pup ratio in the litter [$F(13,54)=1.64$, $p=0.11$] on OTR mRNA levels, whereas this was not the case with the two ER isoforms, and thus litter size and sex ratio were used as covariates for OTR mRNA analysis. Two-way ANOVA revealed a main effect of maternal LG [$F(1,54)=4.64$, $p<0.05$] with a trend for an effect of age [$F(3,54)=2.13$, $p=0.11$] , on relative OTR mRNA levels (**Figure 3.2D**). Subsequent analysis indicated that OTR mRNA levels were elevated among High LG female offspring compared to Low LG female offspring in adulthood [$F(1,13)=6.61$, $p<0.05$], with no significant effect of maternal LG at other developmental time-points.

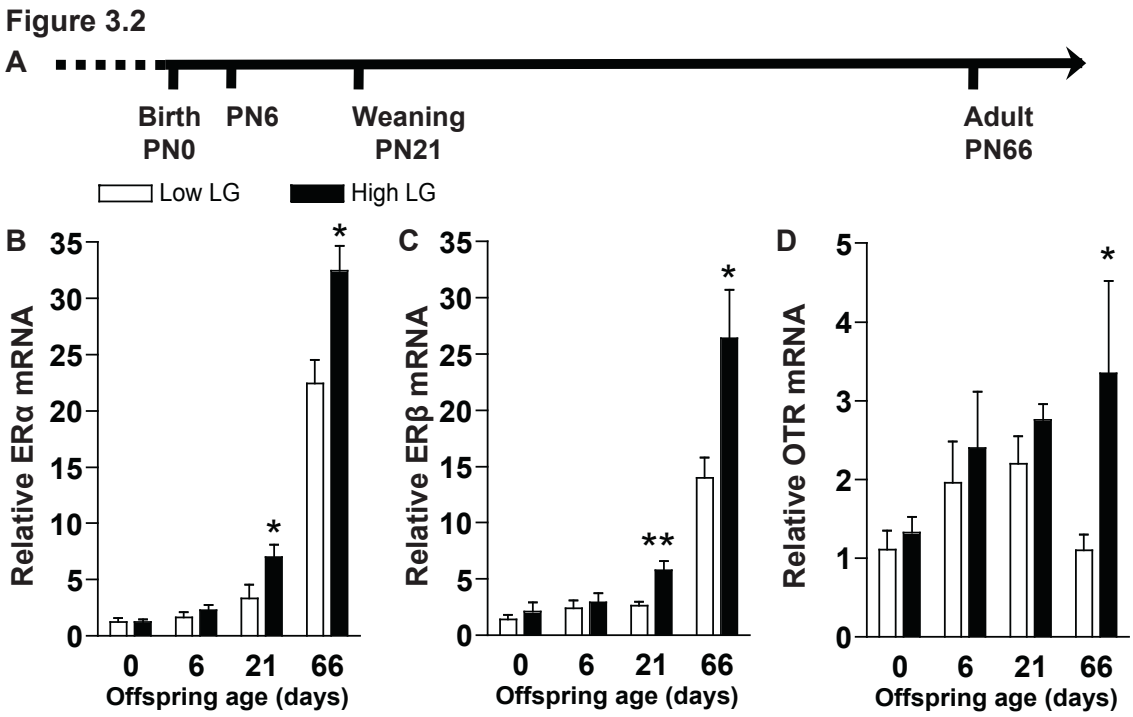


Figure 3.2 Offspring hormone receptor gene expression in the MPOA

(A) Timeline of the four ages at which female offspring of Low LG and High LG dams were taken for gene expression analysis. Mean ± SEM (B) ERα, (C) ERß, and (D) OTR relative mRNA expression, as determined by semi-quantitative real-time PCR, among Low LG and High LG offspring across postnatal development. All relative values were normalized to beta-actin and to the PN0 Low LG group for each transcript examined. *$p<0.05$.

ERα levels in the MPOA of Low and High LG female offspring at PN6

Though group differences in the transcription of *Esr1* (ERα) were not found to be significant until PN21, we hypothesized that LG-associated differences in ERα levels would emerge earlier in development, during the period in which group differences in LG behavior were most pronounced (<PN7), and be specific to neuroanatomical locations within the hypothalamus. In order to explore this possibility with the most anatomical precision, whole brains of PN6 female offspring of Low LG (n=6) and High LG dams (n=5) were fixed, sliced, and immunostained for ERα throughout the hypothalamus. ERα-immunoreactivity (-ir) was

observed in the MPOA, paraventricular nucleus of the hypothalamus, periventricular nucleus of the hypothalamus, arcuate nucleus, and tubercle of PN6 female offspring. When all nuclei were combined in the analysis, there was no effect of maternal LG on ERα cell count or cell density ($p>0.2$; **Figure 3.3A**), a finding consistent with the gene expression data. However, a significant effect of maternal LG was found on the average number of ERα-ir cells within the MPOA [$t(1,9)=2.75$, $p<0.05$; **Figure 3.3B, 3.3D**] as well as cell density [$t(1,9)=2.90$, $p<0.05$] when this brain region was analyzed separately. This effect was not observed in any other nucleus expressing ERα (**Figure 3.3C**), indicating site-specificity of the effect of maternal LG on ERα-ir at this age. There were no differences found in the average size (mm^2) of any nucleus within the hypothalamus between Low LG and High LG offspring ($p>0.2$). We further investigated whether the level of ERα-ir cells was different among Low LG and High LG offspring throughout the MPOA. The MPOA was subdivided into four anterior-posterior sections corresponding to adult relative locations to Bregma: Anterior 1 (.24 to -.12), Anterior 2 (-.12 to -.48), Posterior 1 (-.48 to -.84), and Posterior 2 (-.84 to -1.32). Repeated-measures ANOVA with anterior-posterior section as a within-subject factor and maternal LG as a between-subject factor indicated a significant main effect of LG [$F(1,9)=11.79$, $p<0.01$], section [$F(3,9)=25.53$, $p<0.001$], and a significant interaction between maternal LG and section [$F(3,9)=3.00$, $p<0.05$, sphericity assumed]. Additional analysis indicated a significant effect of maternal LG on the number of ERα-ir cells in the middle two portions of the MPOA [$t(1,9)<2.63$, $p<0.05$], but not the most anterior or the post posterior regions ($p>0.75$), such that females receiving High LG had elevated numbers of ERα-ir cells compared to females experiencing Low LG (**Figure 3.3E**).

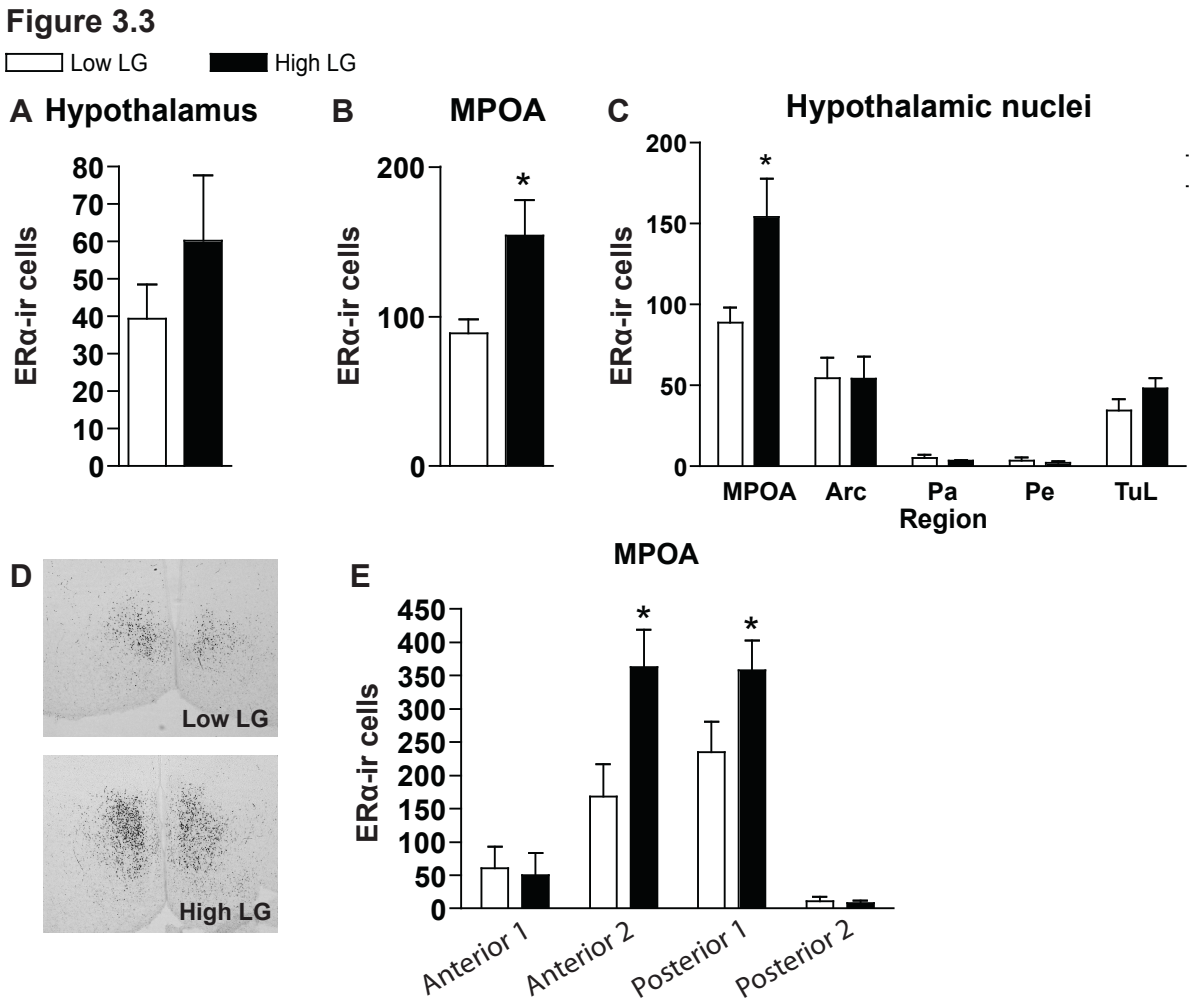


Figure 3.3 PN6 ERα-expressing cells in the hypothalamus

Within Low LG and High LG female offspring at PN6, mean ± SEM number of cells counted expressing ERα protein in (A) total hypothalamus, (B) in the MPOA, and (C) in all hypothalamic regions showing any ERα expression: MPOA, arcuate nucleus (arc), paraventricular nucleus of the hypothalamus (Pa), periventricular nucleus of the hypothalamus (Pe), and lateral tubercle (TuL). (D) Representative images of ERα-ir cells within the MPOA of Low LG and High LG females at PN6. (E) ERα-ir cells in the MPOA, subdivided into four anterior-posterior sections corresponding to adult relative locations to Bregma: Anterior 1 (.24 to -.12), Anterior 2 (-.12 to -.48), Posterior 1 (-.48 to -.84), and Posterior 2 (-.84 to -1.32). *$p<0.05$, **$p<0.01$.

Effects of maternal LG on ERα levels in the MPOA area are sustained into adulthood

The maintenance of group differences in ERα-ir cells in the MPOA through adulthood was determined in adult lactating dams. Among lactating female dams (at postpartum day 6), the average number of ERα-ir cells within the MPOA was significantly elevated in High LG compared to Low LG lactating female offspring [$t(1, 10)=3.28$, $p<0.01$; **Figure 3.4A, 3.4B**]. Repeated measures ANOVA with anterior-posterior section and maternal LG as factors indicated a significant main effect of section [$F(3,14)=7.44$, $p<0.01$] and of maternal LG [$F(1,7)=12.84$, $p<0.01$]. Consistent with the findings among PN6 offspring, ERα-ir cells were elevated within the two middle portions of the MPOA among High compared to Low LG dams ($p<0.05$).

Figure 3.4

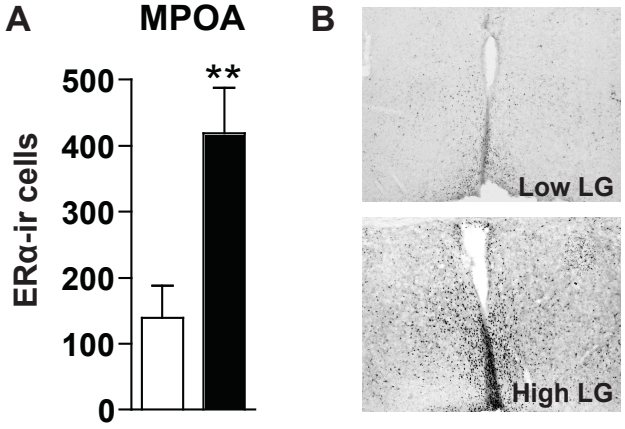


Figure 3.4 ERα-ir cells in the MPOA of lactating dams

(A) Within Low LG and High LG lactating dams at postpartum day 6, mean ± SEM number of cells counted expressing ERα protein in the MPOA. (B) Representative images of ERα-ir cells within the MPOA of Low LG and High LG lactating dams. *$p<0.05$, **$p<0.01$.

Maternal LG alters DNA methylation within the Esr1 regulatory region

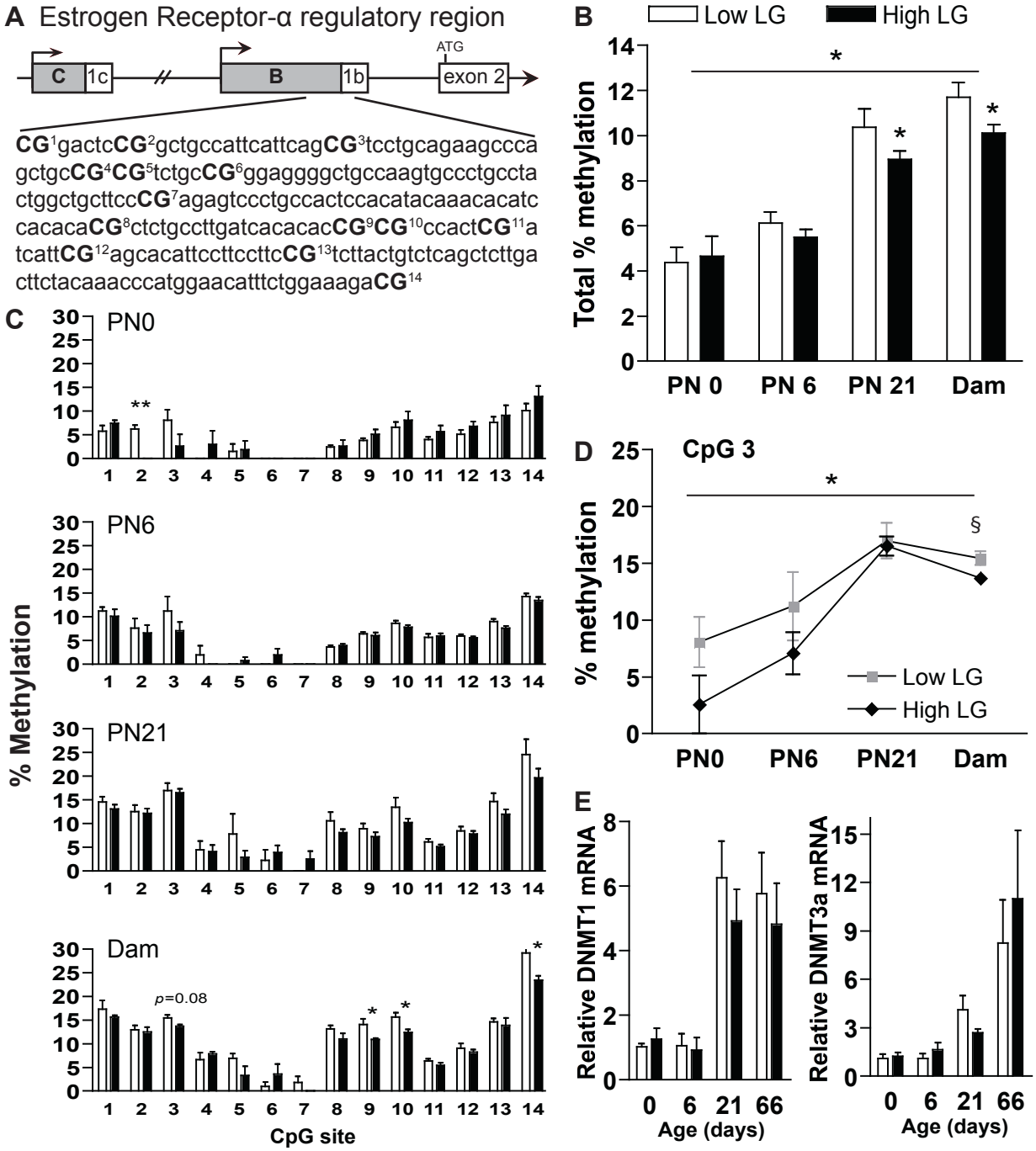
Previous studies have indicated that DNA methylation within the *Esr1* B/1b sequence regulates the expression of this gene, and that variation in postnatal maternal LG can alter DNA methylation of *Esr1* in the hypothalamus of adult female offspring (Champagne et al., 2006). We examined the developmental onset of differences in DNA methylation associated with the experience of Low or High maternal LG across 14 CpG sites in the *Esr1* B/1b regulatory region (**Figure 3.5A**). Three cohorts contributed animals to analysis of *Esr1* methylation; there was no effect of cohort on methylation so the data was pooled. Only samples with high-quality signal were used in the analysis (sample sizes of Low LG and High LG female offspring at each age, respectively: PN1: n=3, n=3; PN6: n=5, n=9; PN21: n=5, n=7; postpartum day 6: n=8, n=4). Analysis of average percent methylation for all CpG sites using two-way ANOVA, with litter as a covariate, revealed a main effect of age [$F(3, 43)=51.41$, $p<0.001$] and of maternal LG [$F(1, 43)=5.86$, $p<0.05$]. Further analysis revealed significantly increased total *Esr1* promoter 1b methylation among Low LG compared to High LG offspring at PN21 [$F(1,11)=5.55$, $p<0.05$; **Figure 3.5B**] and among Low compared to High LG dams at postpartum day 6 [$F(1,11)=8.80$, $p<0.05$]. Significant differences in *Esr1* DNA methylation were not detected at birth or at PN6. These results were confirmed by repeated measures analysis taking into account percent methylation at each of the 14 individual CpG sites with a significant effect of maternal LG at PN21 [$F(1,9)=5.55$, $p<0.05$; **Figure 3.5C**] and at postpartum day 6 [$F(1,9)=8.80$, $p=<0.05$]. DNA methylation levels at CpG3 in *Esr1* B/1b were of particular interest as this site is adjacent to a Stat5b transcription factor binding site. We found a significant effect of maternal LG [$F(1,43)=6.23$, $p<0.05$; **Figure 3.5D**] and of age [$F(3,43)=13.98$, $p<0.001$] on methylation levels at CpG3 in *Esr1* B/1b. We have previously observed region-specific differences in DNA

methylation to correspond to differences in the expression of DNA methyltransferases (Jensen Peña et al., 2012). Comparison of DNMT1 and DNMT3a mRNA levels of Low and High LG female offspring across development (PN0, PN6, PN21, PN66) revealed a significant effect of age but not of maternal LG [age, $F(3,54)>9.48$, $p<0.001$; **Figure 3.5E**].

Figure 3.5 DNA methylation of *Esr1* in offspring of Low and High LG dams

(A) Schematic of the *Esr1* gene regulatory region, including the proximal B promoter and 5'UTR 1b; the first translated exon is Exon 2 (adapted from Freyschuss & Grandien, 1996; Ensembl ENSRNOT00000026350). 14 CpG sites in the B/1b regulatory region were analyzed by bisulfite pyrosequencing and are numbered and indicated in bold. (B) Mean ± SEM total percent methylation from all 14 CpG sites among Low LG and High LG offspring at PN0, PN6, PN21, and among Low LG and High LG dams at postpartum/lactation day 6 (Lac6). (C) Mean ± SEM percent methylation at each CpG site among Low LG and High LG females at each developmental stage. (D) Mean ± SEM percent methylation at CpG3 in the *Esr1* promoter across development. (E) Mean ± SEM *Dnmt1* and *Dnmt3a* relative mRNA expression among Low LG and High LG offspring across postnatal development. All relative values were normalized to beta-actin and to the PN0 Low LG group for each transcript examined. § $p<0.1$, *$p<0.05$, **$p<0.01$, bar indicates a main effect of maternal LG.

Figure 3.5


A Estrogen Receptor-α regulatory region
C
1c
B
1b
ATG
exon 2
CG1gactcCG2gctgccattcattcagCG3tcctgcagaagccca
gctgcCG4CG5tctgcCG6ggaggggctgccaagtgccctgccta
ctggctgcttccCG7agagtccctgccactccacatacaaacacatc
cacacaCG8ctctgccttgatcacacacCG9CG10ccactCG11a
tcattCG12agcacattccttccttcCG13tcttactgtctcagctcttga
cttctacaaacccatggaacatttctggaaagaCG14
B
Low LG
High LG
Total % methylation
PN 0
PN 6
PN 21
Dam
C
PN0
PN6
PN21
Dam
% Methylation
p=0.08
CpG site
D
CpG 3
% methylation
Low LG
High LG
PN0
PN6
PN21
Dam
E
Relative DNMT1 mRNA
Relative DNMT3a mRNA
Age (days)
Age (days)

Developmental timing of maternal LG effects on chromatin remodeling at Esr1

Tri-methylation at two different lysine residues on the histone 3 tail are associated with active (H3K4me3) and repressed (H3K9me3) gene transcription (Santos-Rosa et al., 2002; Schotta et al., 2002; Martin and Zhang, 2005; Stewart et al., 2005). There was a significant effect of maternal LG [$F(1,35)=13.97$, $p<0.001$; **Figure 3.6A**] and a trend for a main effect of age [$F(2,35)=2.90$, $p=0.07$] on the association of H3K4me3 with the *Esr1* B/1b regulatory region. Additional analysis indicated a significant increase in *Esr1* B/1b association with H3K4me3 in High LG compared to Low LG female offspring at PN21 ($p<0.05$) and PN66 ($p<0.05$), with a trend for increased association at PN6 ($p=0.09$). Two-way ANOVA revealed a main effect of maternal LG on association of H3K9me3 with the *Esr1* B/1b regulatory region [$F(1,35)=5.84$, $p<0.05$; **Figure 3.6B**] and with the first translated exon [$F(1,35)=4.38$, $p<0.05$]. At PN21 the association of the *Esr1* B/1b with H3K9me3 was significantly increased in Low LG female offspring compared to High LG offspring ($p<0.05$; **Figure 3.6B**). Differences in chromatin remodeling associated with maternal LG were not detected within a more upstream region in the promoter (**Figure 3.6A-B**).

Figure 3.6

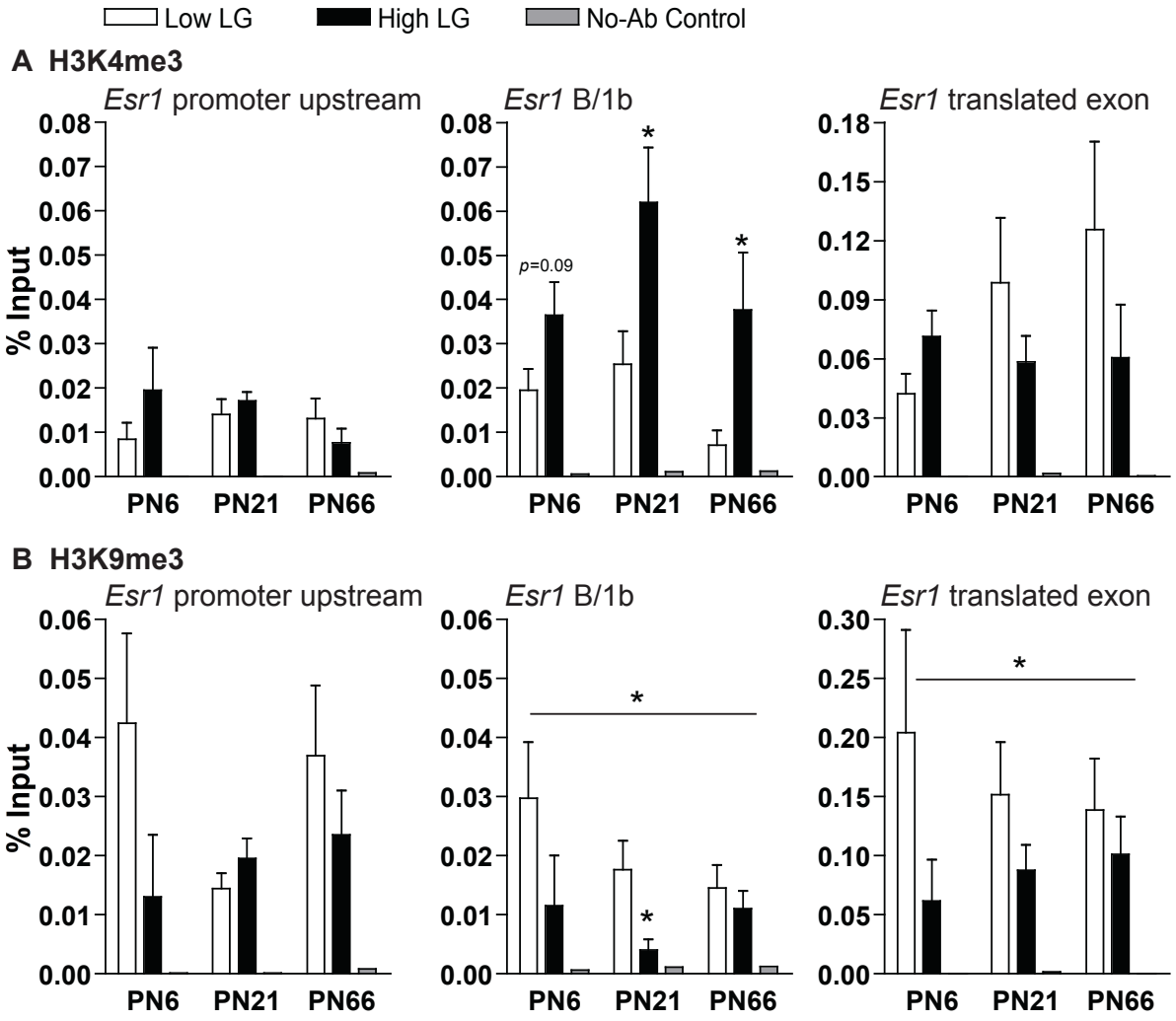


Figure 3.6 Post-translational histone modifications associated with offspring *Esr1*

Chromatin immunoprecipitation of the *Esr1* B/1b regulatory region by antibodies against (A) trimethylated histone H3 lysine 4 (H3K4me3) or (B) trimethylated histone H3 lysine 9 (H3K9me3). Three regions of the *Esr1* gene were examined for association with each antibody: the B promoter (-219 to -103), the B/1b regulatory region including 6 of the 14 CpG sites analyzed for methylation (+22 to +151), and within the first translated exon (exon 2, +2246 to +2479). The B/1b regulatory region amplified (+22 to +151) includes 6 of the 14 CpG sites analyzed for methylation. Mean ± SEM percent of input among Low LG and High LG female offspring at PN6, PN21, and PN66. *$p<0.05$, bar indicates main effect of maternal care across ages.

Postnatal maternal LG predicts maternal sensitization in juvenile female offspring

Maternal sensitization testing of juvenile (PN28-40) female offspring of Low LG and High LG dams indicated that the latency to onset of full maternal behavior (including retrieval of all 3 pups to the nest, crouching over pups, and licking/grooming) was significantly shorter in female offspring of High compared to Low LG dams [$t(1,22)=2.36$, $p<0.05$; **Figure 3.7A**]. This finding was confirmed using Kaplan-Meier survival analysis [Generalized Wilcoxon test, $X^2(1, 22)=4.88$, $p<0.05$; **Figure 3.7B**].

Figure 3.7

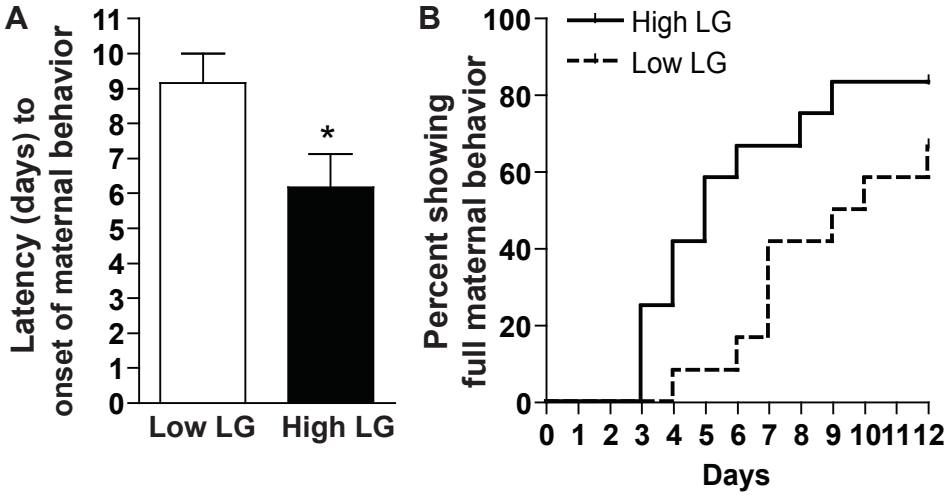


Figure 3.7 Maternal sensitization latency among Low and High LG offspring

(A) Mean ± SEM latency of Low LG or High LG juvenile offspring to maternal responsivity. Latency was determined as the number of days until a female, exposed to new 3 freshly nursed young donor pups each day in her home cage, retrieved all 3 pups to a nest, crouched, and groomed the pups. (B) Survival analysis showing percent of Low LG and High LG female offspring from the first day of pup exposure until fully maternally sensitized or the end of testing at 12 days. *$p<0.05$.

Sensitive periods for maternal LG-associated variation in maternal sensitization

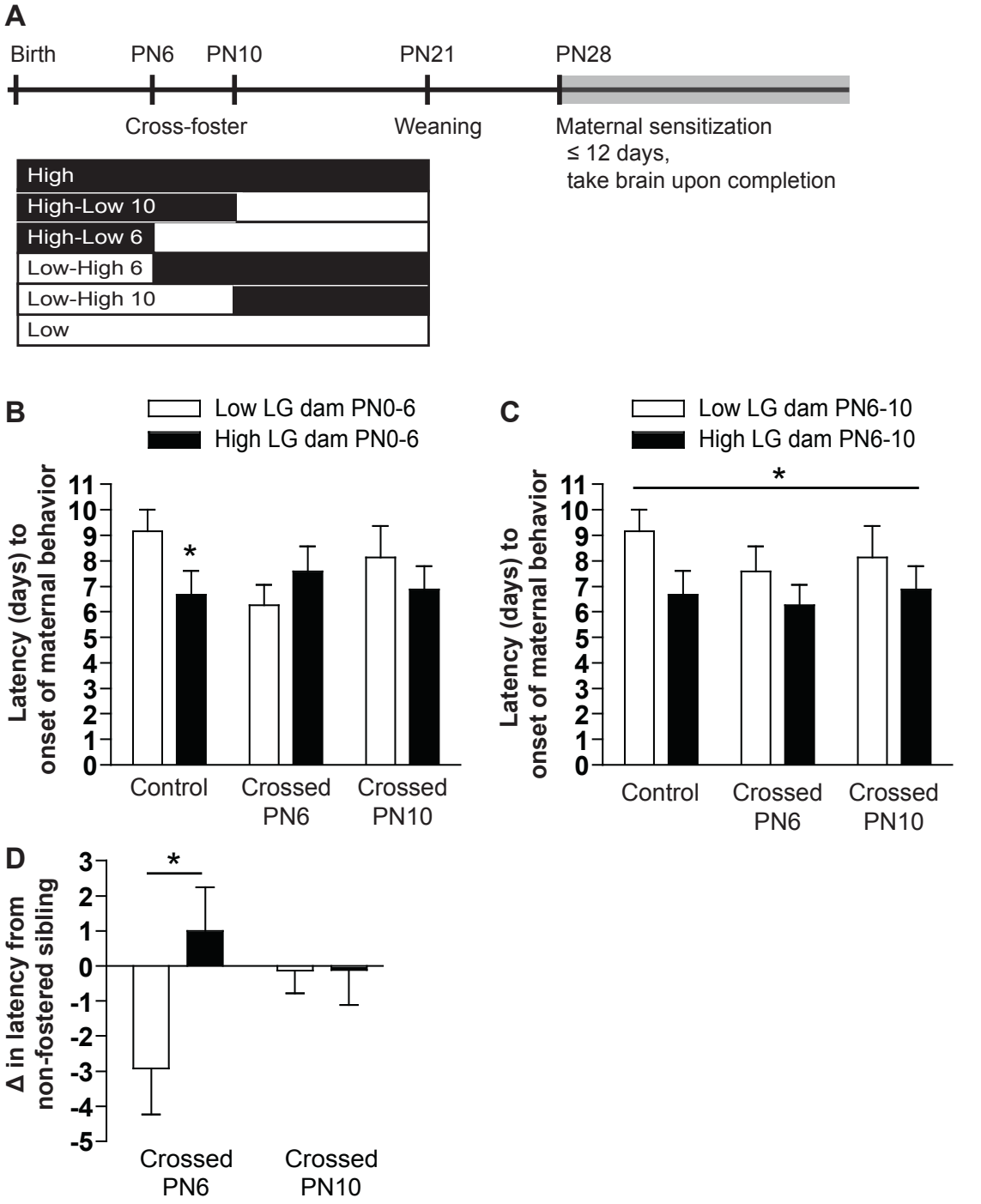
To determine whether maternal sensitization latency is sensitive to maternal LG experienced at different time points in the pre-weaning period, female offspring were cross-fostered between Low LG and High LG dams at PN6 or at PN10 (**Figure 3.8A**) and tested for juvenile maternal sensitization latency. Multiple ANOVA with initial rearing condition (Low/High LG), whether the pup was cross-fostered at PN6, and whether cross-fostered at PN10 as independent factors revealed that the effect of initial rearing condition (PN1-6) on offspring maternal sensitization latency is significantly altered by cross-fostering at PN6 [initial rearing condition by PN6 fostering interaction: $F(1,62)=6.60$, $p<0.05$; **Figure 3.8B**], but not by cross-fostering at PN10 (initial rearing condition by PN10 fostering: $p>0.8$). As cross-fostering at PN6 produced an altered behavioral phenotype, but cross-fostering at PN10 did not, we subsequently determined whether there was an effect of the maternal LG experienced between PN6-10 on offspring maternal sensitization latency. ANOVA with PN6-10 rearing condition (Low/High LG), whether the pup was cross-fostered at PN6, and whether cross-fostered at PN10 as independent factors revealed a main effect of PN6-10 maternal care on offspring maternal sensitization latency [$F(1,62)=4.15$, $p<0.05$; **Figure 3.8C**]. Subsequent analyses indicated that regardless of what rearing condition preceded or followed, rearing by a High LG dam from PN6-10 significantly reduced maternal sensitization latency [main effect of rearing condition PN6-10: $F(1,62)=7.07$, $p<0.01$; **Figure 3.8C**]. In addition, because all cross-fostered animals had a non-cross-fostered sibling, we were able to compare the change in maternal sensitization behavior in the fostered *vs.* non-fostered sibling (**Figure 3.8D**). Multiple ANOVA (with initial rearing group, PN6 fostered, and PN10 fostered as fixed factors) revealed an interaction between initial dam and cross-fostering at PN6 [$F(1, 62)=5.22$, $p<0.05$] but no interaction with cross-fostering at PN10. Taken together, these analyses indicate that cross-fostering female offspring at PN10

between Low LG and High LG dams has no significant effect on maternal sensitivity, while cross-fostering females at PN6 produces a shift in maternal sensitivity corresponding to the rearing condition provided by the adoptive dam.

Figure 3.8 Maternal sensitization latency among offspring cross-fostered between Low and High LG dams in the postnatal period

(A) Timeline of cross-fostering and maternal sensitization testing and the groups created. Offspring were cross-fostered between Low LG and High LG dams at PN6, PN10, or were non-fostered controls. All animals were weaned at PN21 into single housing and maternal sensitization testing began at PN28. Six groups were created by cross-fostering: control Low, control High, cross-fostered at PN6 ("Low-High 6" and "High-Low 6"), and cross-fostered at PN10 ("Low-High 10" and "High-Low 10"). (B) Mean ± SEM latency to maternal sensitization, based on initial dam (PN0-6), and age of cross-fostering or control. There was a significant interaction between being cross-fostered on PN6 and initial maternal LG on latency to maternal sensitization. (C) As in B, but group is based on LG status of the dam experienced between PN6-10. There was a main effect of dam from PN6-10 on latency to maternal sensitization after controlling for cross-fostering. (D) Siblings that were cross-fostered at PN6 or PN10 were compared to their non-fostered sibling on latency to maternal sensitization. *$p<0.05$.

Figure 3.8


A
Birth
PN6
PN10
PN21
PN28
Cross-foster
Weaning
Maternal sensitization
≤ 12 days,
take brain upon completion
High
High-Low 10
High-Low 6
Low-High 6
Low-High 10
Low
B
Low LG dam PN0-6
High LG dam PN0-6
Latency (days) to onset of maternal behavior
Control
Crossed PN6
Crossed PN10
C
Low LG dam PN6-10
High LG dam PN6-10
Latency (days) to onset of maternal behavior
Control
Crossed PN6
Crossed PN10
D
Δ in latency from non-fostered sibling
Crossed PN6
Crossed PN10

Sensitive periods for maternal LG-associated variation in gene expression

We next examined whether the changes in maternal sensitivity observed following cross-fostering were accompanied by changes in hormone receptor mRNA levels in the MPOA. Within the control non-cross-fostered group a significant difference in ERα mRNA levels by initial rearing condition was confirmed [$t(1,10)=3.53$, $p<0.01$]. Multiple ANOVA revealed a significant main effect of initial rearing condition on relative ERα mRNA [$F(1,36)=5.44$, $p<0.05$; **Figure 3.9A**], as well as a significant interaction between initial rearing condition and cross-fostering at PN6 [$F(1,36)=8.27$, $p<0.01$] but no interaction between initial rearing condition and PN10 cross-fostering ($p>0.5$). There was also a main effect of maternal LG from PN6-10 on offspring ERα mRNA levels [$F(1,36)=9.54$, $p<0.01$], and an interaction between maternal LG from PN6-10 and being cross-fostered at PN6 [$F(1,36)=4.15$, $p<0.05$]. We also examined the change in relative ERα levels in fostered compared to non-fostered siblings (**Figure 3.9B**). Multiple ANOVA (with initial rearing condition, PN6 fostered, and PN10 fostered as fixed factors) revealed a trend for an interaction between initial rearing condition and being cross-fostered at PN6 [$F(1, 29)=3.77$, $p=0.06$] but no interaction with being cross-fostered at PN10 ($p>0.9$) on change in ERα mRNA levels. Taken together, these results parallel the observed effects of cross-fostering on behavior in that fostering at PN10 has no significant effect on MPOA ERα mRNA levels, while fostering at PN6 produces a shift in ERα mRNA levels towards that of the condition provided by the adoptive dam.

There was a significant effect of initial rearing condition on relative ERß mRNA levels among non-cross-fostered controls [$t(1,10)=2.35$, $p<0.05$; **Figure 3.9C**], but no significant group differences were found after cross fostering at either age ($p>0.4$). This finding suggests that ERß mRNA levels are sensitive to levels of maternal LG occurring beyond PN10. In addition to a significant effect of initial rearing condition among non-cross-fostered controls on OTR mRNA

levels [$t(1,10)=2.96$, $p<0.05$; **Figure 3.9D**], there was also a trend for a main effect of initial rearing condition on OTR mRNA levels across all groups [$F(1,35)=3.88$, $p=0.06$] and cross-fostering at either age did not significantly impact this initial rearing experience effect ($p>0.6$; **Figure 3.9D**). Collapsing by initial maternal LG group and controlling for cross-fostering as covariates, there was a significant effect of initial PN 0-6 maternal LG on OTR levels [$F(1,35)=4.69$, $p<0.05$]. These results suggest that OTR mRNA levels are sensitive to maternal LG experienced before but not after PN6.

Figure 3.9

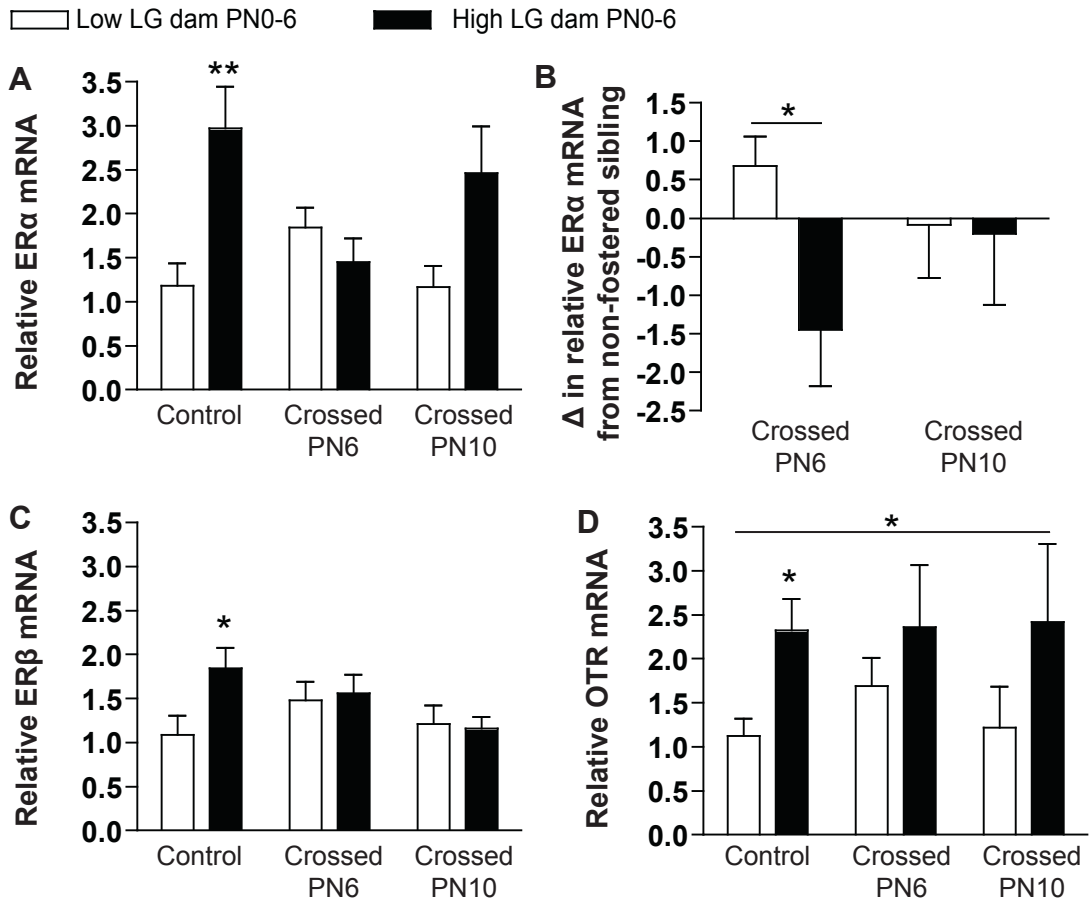


Figure 3.9 Hormone receptor gene expression in the MPOA among offspring cross-fostered between Low and High LG dams in the postnatal period

Mean ± SEM relative (A) ERα, (C) ERß, and (D) OTR mRNA levels among females reared initially by a Low LG or High LG dam from PN0-6 and that were non-fostered controls, cross-fostered at PN6, or cross-fostered at PN10. (B) Mean ± SEM of the difference in relative ERα mRNA levels between control siblings and those cross-fostered at PN6 or PN10. All relative values were normalized to beta-actin and to the Control Low LG group for each transcript examined. *$p<0.05$.

Discussion

Though maternal LG experienced during the postnatal period has previously been shown to alter estrogen receptor mRNA and protein levels, CpG methylation in the *Esr1* gene promoter, and postpartum maternal behavior in adult female offspring, the developmental timing of these effects had yet to be elucidated. Here we demonstrate that maternal LG is associated with the emergence of changes in the expression of hormone receptors (ERα, ERβ, OTR) in the developing hypothalamus and in the case of ERα, group differences in transcriptional activity are associated with variation in DNA methylation and histone modifications within the *Esr1* gene regulatory region. These epigenetic and transcriptional effects are apparent by PN21 and in the current study we identify a parallel behavioral to these molecular changes: variation in the maternal sensitization of juveniles. Moreover, we provide evidence for a critical period in the emergence of maternal LG-associated effects on juvenile maternal sensitivity and hypothalamic gene expression, which limits the plasticity of these outcomes beyond PN10. Overall, these studies illustrate the molecular and behavioral pathways that link the experience of maternal care during infancy to variation in maternal behavior exhibited in adulthood, thus demonstrating a mechanism of the behavioral transmission of maternal behavior and variation in the maternal brain across generations.

Estrogen sensitivity is a critical feature of the neuroendocrine regulation of maternal behavior. This sensitivity is mediated in large part through ligand-dependent interactions with estrogen receptors and consequent activation of transcription through estrogen response elements in target genes (Fahrbach and Pfaff, 1986; Ahdieh et al., 1987; Bale and Dorsa, 1997). ERα knockout mice exhibit significant deficits in pup retrieval and increased rates of infanticide (Ogawa et al., 1998). Targeted reductions of ERα specifically within the MPOA by shRNA

(Spiteri et al., 2012) or RNAi (Spiteri et al., 2012) nearly abolished maternal behaviors, including pup retrieval, LG, and nursing. The reduced estrogen-sensitivity previously observed in the offspring of Low LG dams is likely mediated by the reduced levels of hypothalamic ERα evident in these females. Our analyses of the effect of LG during development indicate that both ER isoforms are reduced in expression at PN21 among Low LG offspring, and thus these group differences emerge during the period of mother-pup interactions. Likewise, we found longer latencies to onset of maternal behavior among Low LG females in the juvenile period, indicating that differences in both estrogen sensitivity and maternal behavior are detectable at a young age. These effects may or may not be mediated by offspring variation in OTR, as we did not find significant group differences in OTR mRNA until adulthood. Previous studies indicate that OTR expression significantly increases in the hypothalamus and olfactory tubercle during puberty (Gimpl and Fahrenholz, 2001), and this developmental period may be a critical time for ER-hormone interactions. Thus, puberty may be a significant trigger in the OTR expression differences among offspring of Low and High LG dams and account for the delay in observed group differences in OTR.

The time-course of changes in ERs and OTR levels found here in response to maternal behavior is consistent with previous research on the levels of these hormone receptors across development. Previous studies have found a critical period for estrogen to masculinize sexually dimorphic brain region morphology, including the MPOA, from E18 to postnatal day 5, which is consistent with the timing of ER development and environmentally induced alterations ERα-ir found at PN6 in these studies (Rhees et al., 1990a; Rhees et al., 1990b; DonCarlos and Handa, 1994; Schwarz and Mccarthy, 2008). Our findings are consistent with previous literature showing the distribution of hypothalamic ERα-ir cells to be similar among neonates and adults

(Yokosuka et al., 1997), and that ER levels increase significantly in the hypothalamus and amygdala during puberty (Brown et al., 1994). Pregnancy and parturition also mark significant changes in central estrogen and oxytocin receptors. OTR mRNA and activated ER binding to DNA increases throughout pregnancy and remains elevated during lactation, particularly in the MPOA, concurrent with increased responsiveness towards pups (Rosenblatt et al., 1994; Rosenblatt et al., 1998; Meddle et al., 2007). While studies have explored levels of these hormone receptors across development generally and in response to neonatal steroid hormone treatment, this is the first study to investigate the developmental time course of individual variation in female hormone receptor levels, important for variation in maternal behaviors, in response to variation in maternal LG.

Though changes in ERα mRNA associated with the expression of maternal LG were not detected until PN21, elevated ERα-ir cells within the MPOA among High compared to Low LG offspring were detected as early as PN6. Previous studies, using in situ hybridization, have indicated that mRNA levels within the MPOA can be detected as early as PN6 (Champagne et al., 2006), suggesting that the anatomical specificity of maternal care effects on ERα may limit detection of these effects when using techniques that pool tissue across hypothalamic subregions. While several hypothalamic nuclei exhibit differences in OTR binding among adult High and Low LG offspring (Champagne et al., 2001), maternal LG-induced variation in ERα protein appear to be highly specific to the MPOA, and to specific subregions within the MPOA. However, there are other possible explanations for the lack of concordance in ERα mRNA and protein levels. High immunoreactivity corresponding to low mRNA levels has been previously reported (Chen et al., 2002; Lichtinghagen et al., 2002; Duchrow et al., 2003; Greenbaum et al., 2003; Dickson et al., 2007) and thus it may also be possible that while the number of ERα-ir

cells is elevated among High LG offspring, the overall levels of mRNA produced by the cells is not different at PN6. Whether this discrepancy is due to methodological sensitivity, or post-transcriptional modulation, for example by microRNAs (Hah et al., 2011; Morgan and Bale, 2011), will be an interesting phenomenon to explore further.

There is increasing evidence for epigenetic regulation of ERα levels in the brain through variation in DNA methylation of the regulatory region of *Esr1* associated with developmental experiences. Treatment of PN0 pups with estradiol (100μg s.c.) revealed modest changes in MPOA *Esr1* intron methylation over development at PN1, PN20, and PN60 compared to control, and alterations were restricted to specific CpG sites (Schwarz et al., 2010), consistent with our current findings. The critical role of LG as opposed to other aspects of maternal behavior in modulating *Esr1* methtlayion is suggested by studies manipulating neonatal tactile stimulation. Simulated maternal grooming (additional paintbrush stimulation of the anogential region by investigators, performed PN5-7) increases rat *Esr1* promoter B/1b methylation compared to control females at two CpG sites, concurrent with decreases in relative ERα mRNA (Kurian et al., 2010). Increased CpG methylation within the *Esr1* B/1b regulatory region was also previously shown in adult Low LG female offspring as one potential mechanism of decreased gene expression (Champagne et al., 2006). The current studies show an effect of maternal LG on *Esr1* B/1b methylation by PN21, consistent with the developmental timing of group differences in mRNA expression, and provide a critical missing link in understanding mechanisms leading from experience of maternal LG to variation in ERα expression and adult maternal behavior.

Previous studies have illustrated environmental modulation of H3K4 and H3K9 methylation levels. Chronic early life stress induced by maternal separation from PN2-9 decreased total H3K9 mono and tri-methylation in the frontal cortex of mice, but association of

these epigenetic marks with specific gene targets was not explored (Kao et al., 2012). Three to four weeks of enriched social and physical environments increased mouse hippocampal BDNF mRNA and H3K4me3 at the BDNF P3 and P6 promoters, while simultaneously decreasing H3K9me3 and H3K37me3 at the BDNF P3 and P4 promoters (Kuzumaki et al., 2011). Here we report increased H3K4me3 and decreased H3K4me3 association with the *Esr1* B/1b regulatory region of High LG females at PN21, consistent with chromatin remodeling to enhance gene expression (Jenuwein and Allis, 2001; Khorasanizadeh, 2004). The evidence for postnatal ERα regulation by histone remodeling presented here would be strengthened by additional ChIP experiments examining RNA polymerase II association with the ERα gene. This epigenetic facilitation of *Esr1* transcription by H3K4me3 appears to emerge during the pre-weaning period and is stable through adulthood, while repression by H3K9me3 may be specific to the early juvenile period, consistent with emergence of variation in mRNA. Together, the data suggest that multiple forms of epigenetic regulation may work in concert to regulate ERα levels in the MPOA. These results are significant because they show epigenetic regulatory mechanisms emerging during the postnatal time period of mother-infant interactions.

Establishing the causality of maternal LG effects on development and regulation of hypothalamic hormone receptor systems is a critical issue to address. Though strong linear correlations between maternal LG and various outcomes (Liu et al., 1997; Champagne et al., 2003a; Cavigelli et al., 2010; Hasselt et al., 2012; Parent et al., 2012) is suggestive of this causal influence, cross-fostering studies have provided the strongest support for the hypothesis that LG induces neurobehavioral changes in offspring. Cross-fostering pups between Low and High LG dams on the day of birth has indicated that adult ERα levels and maternal behavior are associated with the quality of maternal LG experience during postnatal development (Champagne et al.,

2006). Cross-fostering at PN6 or PN10 in the current studies demonstrates that both MPOA ERα mRNA levels and maternal sensitization behavior have continued sensitivity to maternal LG through PN10, despite diminishing variation in maternal LG frequency among High and Low LG dams. It may be argued that PN10 marks the end of sensitivity to maternal LG because there is reduced pup LG after this age. However, tactile stimulation in the form of handling has comparable effects on altered stress physiology in rodents (adrenal ascorbic acid levels, (Levine and Lewis, 1959), and hippocampal GR binding, (Meaney and Aitken, 1985) when administered in the first postnatal week or continually in the pre-weaning period, but not when handling was only administered in the second or third postnatal week. Together these studies argue for a critical period of sensitivity to tactile stimulation, here in the form of LG. While further studies are needed to pinpoint an exact critical period for the effects of maternal LG on development of ERα levels and maternal behavior, our data suggest plasticity of this system in response to maternal care is limited beyond PN10.

Chapter 4: Maternal care shapes development of offspring midbrain dopamine pathways and reward-directed behavior

Introduction

The dopaminergic system is critically involved in reward, motivation, stress, affect, memory, and motor behaviors (see for example Wooten and Trugman, 1989; Wise, 2002, 2004; Rodrigues et al., 2011; Trainor, 2011). Dysfunction in dopamine pathways or degeneration of dopamine neurons in humans are also associated with disorders such as addiction, attention deficit hyperactivity disorder, Tourette syndrome, schizophrenia, and Parkinson's disease (Agid et al., 1989; Koob and Nestler, 1997; Goto and Grace, 2007; Genro et al., 2010; McNaught and Mink, 2011; Rodrigues et al., 2011). The mesolimbic dopamine system is not considered fully mature until the third postnatal week in rodents (Voorn et al., 1988). As such, early life experiences have great potential to shape dopamine system development.

Previous studies have shown that stressful experiences in the prenatal and early postnatal period alter dopaminergic function (Alonso et al., 1994; Henry et al., 1995; Ortiz et al., 1996; Barros et al., 2004; Mcarthur et al., 2005; Jahng et al., 2010; Rodrigues et al., 2011; Baier et al., 2012; Huppertz-Kessler et al., 2012; Ventura et al., 2012). Manipulation of the maternal environment likewise has been found to impact dopamine system development and reward-directed behaviors. Postnatal motherless rearing in rats was found to increase basal NAc dopamine, while the level of dopamine release in response to stimuli was dependent on the

specific stimulus (increased dopamine release in response to food, decreased in response to pups; (Afonso et al., 2011). An unstable maternal environment, created by repeated cross-fostering from PN1-4, was found to reduce preference for palatable food stimuli (Ventura et al., 2012). Together these studies highlight that molecular alterations to the dopamine system and associated behavioral alterations consequent to these changes can emerge during early development and last well into adulthood. While previous studies have focused on stressful manipulations to the postnatal environment, we hypothesized that natural variation in postnatal maternal care, in the form of maternal LG, may also induce long-lasting alterations in midbrain dopamine system development.

Maternal LG-induced variation in offspring mesolimbic dopamine system development is also suggested as one potential mechanism underlying the transmission of maternal behavior from mothers to female offspring. Maternal behavior is a motivated behavior, mediated by dopaminergic signaling in the VTA and NAc, and variation in maternal LG was found to predict dopamine release and receptor binding in the NAc shell of dams (Gaffori and Le Moal, 1979; Giordano et al., 1990; Hansen et al., 1991; Stern and Taylor, 1991; Hansen et al., 1993; Champagne et al., 2004; Numan and Stolzenberg, 2009). The studies in this chapter sought to elucidate whether variation in rat maternal LG predicted variation in development of the mesolimbic dopamine system among female offspring, the time course of these postnatal developmental alterations, and the relevance for natural reward-directed behaviors.

Results

Postnatal maternal behavior

In all cohorts, no differences were found in average number of pups per litter or male/female pup ratio among Low LG compared to High LG dams ($p>0.6$). LG frequency was significantly reduced among Low and High LG dams during the postpartum period [representative cohort with litters used for gene expression and methylation analysis: $t(1,13)=8.23$, $p<0.001$]. Repeated measures analysis with postpartum day as a within-subject factor and maternal LG as a between-subject factor indicated a significant effect of day [$F(5,65)=6.54$, $p<0.001$] and a significant effect of maternal LG [$F(1,13)=50.74$, $p<0.001$], but not a significant interaction between the two ($p>0.21$). Consistent with previous studies (Jensen Peña et al., Behavioral Neuroscience, in press), LG decreased across postpartum days in both Low LG and High LG dams and group differences in LG were apparent across PN1-6.

Maternal behavior influences tyrosine hydroxylase (TH) immunoreactivity in the VTA

In the VTA of PN6 female offspring, a significant effect of maternal LG was found on the average number of cells expressing TH [$t(1,9)=3.13$, $p<0.05$; **Figure** 4.**1A, 4.1C**] and on cell density [$t(1,9)=2.90$, $p<0.05$], such that female offspring reared by High LG dams had elevated levels of TH-immunoreactive (-ir) cells compared to females reared by Low LG dams. This effect was not statistically significant within the substantia nigra (SN; **Figure 4.1B, 4.1C**) No significant differences were found in the size of the VTA or SN ($p>0.36$) at PN6.

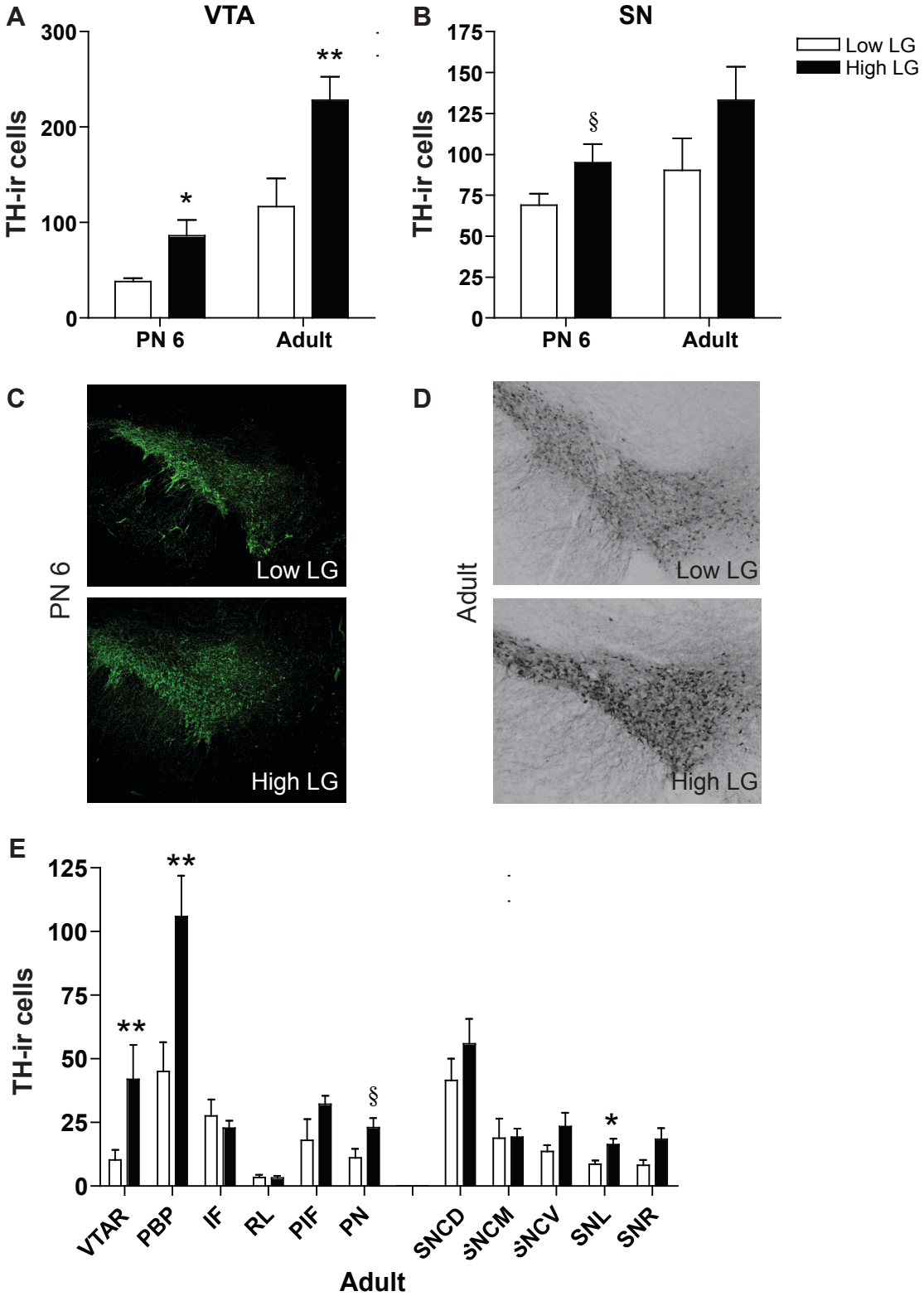
In adult female offspring, cycle state was used as a covariate in the analysis. There was a significant effect of maternal LG group on TH-ir in the VTA [$F(1,11)=11.32$, $p<0.01$, **Figure** 4.**1A, 4.1D**] such that High LG adult female offspring had elevated levels of TH-ir cells

compared to Low LG females. Similar to the findings in PN6 offspring, there was no significant effect of maternal LG on TH-ir in the SN (**Figure** 4.**1B, 4.1D**). To determine the anatomical localization of the effects of maternal LG in the adult brain, we determined TH-ir in specific nuclei within the VTA and SN. The number of TH-ir cells was significantly greater among High LG compared to Low LG adult offspring in the PBP [$F(1, 11)=9.73$, $p<0.01$; **Figure** 4.**1E**], VTAR [$F(1, 11)=12.75$, $p<0.01$], and SNL [$F(1, 11)=7.12$, $p<0.05$], with a trend in the PN [$F(1, 11)=4.00$, $p=0.08$], and no significant effects found in any other region.

Figure 4.1 TH-ir cells in the ventral tegmental area and substantia nigra of PN6 and adult offspring

Within Low LG and High LG female offspring at PN6 and as adults, mean ± SEM number of cells counted expressing TH protein in the total (A) ventral tegmental area and (B) substantia nigra. Representative images of TH-ir cells within the ventral midbrain of Low LG and High LG females at (C) PN6 and (D) in adulthood. (E) Within adult Low and High LG offspring, mean ± SEM number of cells counted expressing TH protein in individual nuclei of the VTA and SN: rostral VTA (VTAR; parafasciculus retroflexus area by Ikemoto 2007), parabrachial pigmented nucleus of the VTA (PBP), paranigral nucleus of the VTA (PN), parainterfascicular nucleus of the VTA (PIF), rostral linear nucleus of the raphe (RLi), caudal linear nucleus of the raphe (CLi), interfascicular nucleus (IF), and the substantia nigra pars compacta dorsal tier (SNCD), medial tier (SNCM), ventral tier (SNCV), lateral part (SNL), and reticular part (SNR). § $p<0.1$, *$p<0.05$, **$p<0.01$.

Figure 4.1


A
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Adult

Maternal LG alters the expression of genes critical for midbrain dopamine neuron differentiation and maintenance

The differential TH cell counts within the VTA associated with variation in postnatal maternal care suggest that the development of dopaminergic pathways are shaped be maternal LG. To examine the potential mechanisms of this effect, we determined the expression during postnatal development (PN0, PN6, PN21, PN66) of transcription factors that regulate the maturation of the dopaminergic system, including Nurr1 (Nr4a2), Cdkn1c (cyclin-dependent kinase inhibitor n1c, $p57^{kip2}$), Lmx1b (of the LIM homeodomain family), Pitx3 (paired-like homeodomain transcription factor), and BNDF (brain derived neurotrophic factor). Nurr1 and Cdkn1c interact in dopamine neuron differentiation. Lmx1b and Pitx3 are involved in dopamine maintenance and survival, and BDNF is a secreted neurotrophic factor additionally involved in reward and motivation. We found a main effect of age on *Nurr1* expression [$F(3,53)=39.24$, $p<0.001$; **Figure 4.2A**] with relative mRNA levels decreasing with age, but not a significant main effect of maternal LG [$F(1,53)=2.21$, $p=0.14$]. There was also a main effect of age on *Cdkn1c* [$F(3,53)=18.96$, $p<0.001$], and a trend for a main effect of maternal LG [$F(1,53)=2.95$, $p=0.09$; **Figure 4.2B**]. Further analysis revealed a significant difference in *Cdkn1c* expression at PN6 [$t(1, 11)=2.42$, $p<0.05$], such that Low LG offspring expressed lower levels of *Cdkn1c* compared to High LG offspring. Two-way ANOVA revealed a significant main effect of age [$F(3,53)=14.10$, $p<0.001$] and a main effect of maternal LG [$F(1,53)=8.46$, $p<0.01$; **Figure 4.2C**] on *Lmx1b* mRNA across postnatal development. *Lmx1b* mRNA was elevated among High LG compared to Low LG female offspring at PN21 [$t(1, 12)=2.45$, $p<0.05$] and PN66 [$t(1, 12)=3.00$, $p<0.05$]. There was a main effect of age [$F(3,50)=3.98$, $p<0.05$; **Figure 4.2D**] and a trend for an effect of maternal LG [$F(1,50)=3.85$, $p=0.06$] on relative *Pitx3* mRNA levels, due

primarily to elevations in *Pitx3* mRNA in High LG female offspring in adulthood. Two-way ANOVA revealed a main effect of maternal LG [$F(1,53)=7.75$, $p<0.01$; **Figure 4.2E**] but not of age [$F(3,53)=2.09$, $p=0.12$] on relative VTA *Bdnf* mRNA levels, due primarily to elevations in *Bdnf* mRNA in adult High LG offspring ($p<0.05$).

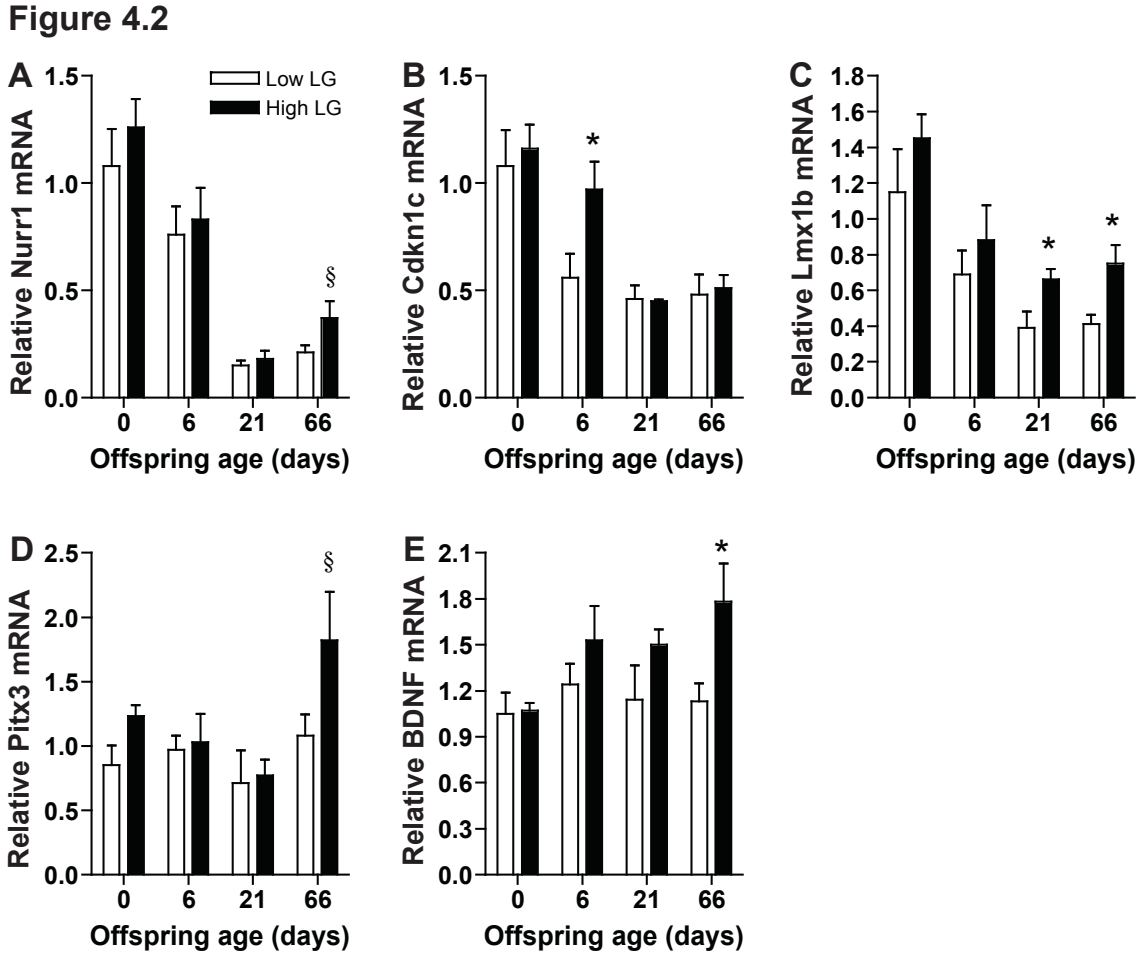

Figure 4.2 Offspring gene expression in the ventral midbrain

Mean ± SEM relative mRNA expression of (A) *Nurr1*, (B) *Cdkn1c*, (C) *Lmx1b*, (D) *Pitx3*, and (E) *Bdnf,* as determined by semi-quantitative real-time PCR, among Low LG and High LG offspring across postnatal development (n=6-8 per group per age). All relative values were normalized to beta-actin and to the PN0 Low LG group for each transcript examined. § $p<0.1$, *$p<0.05$.

Maternal LG affects dopamine receptor gene expression in the NAc during development

Dopamine neurons of the VTA involved in reward and motivated behaviors project to the ventral striatum (Björklund and Dunnett, 2007). To determine if maternal LG also influenced development of the mesolimbic dopamine system at the level of dopamine receptors, we analyzed relative mRNA levels of dopamine receptors D1, D2, and D3, as well as BDNF and TrkB in the ventral striatum. There was a main effect of age on levels of the three dopamine receptors examined [$F(3,52)>15.47$, $p<0.001$; **Figure 4.3A-C**]. Analysis of *Drd1* expression indicated an interaction between age and maternal LG [$F(3,52)=2.73$, $p=0.06$], due primarily to significantly increased *Drd1* mRNA amongst High LG offspring at PN21 [$t(1, 12)=2.51$, $p<0.05$; **Figure 4.3A**]. A similar pattern of findings was evident for *Drd2* [age by maternal LG interaction: $F(3,52)=3.18$, $p<0.05$; **Figure 4.3B**] and *Drd3* [age by maternal LG interaction: $F(3,52)=3.56$, $p<0.05$; **Figure 4.3B**] with elevations in *Drd2* and *Drd3* at PN21 among High LG female offspring ($p<.05$). We also examined the effect of maternal LG on levels of *Bdnf* and its receptor *Trkb* in the NAc across development. Two-way ANOVA revealed a main effect of age [$F(3,52)=5.08$, $p<0.01$; **Figure 4.3D**] but not maternal LG ($p=0.21$) on *Bdnf*. Similarly, for *Trkb*, there was a main effect of age [$F(3,52)=9.58$, $p<0.001$] but not of maternal LG [$F(3,52)=2.35$, $p=0.08$; **Figure 4.3E**].

Figure 4.3

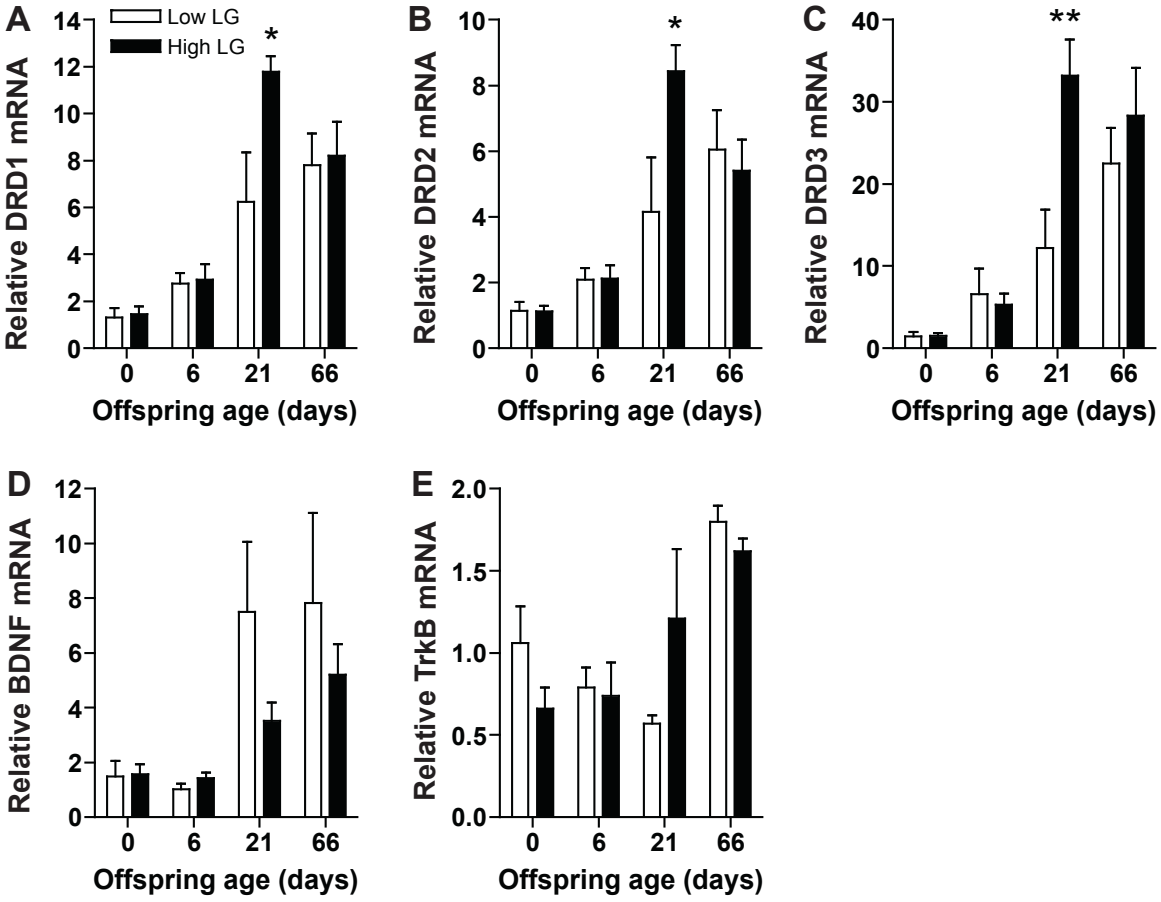

Figure 4.3 Offspring gene expression in the nucleus accumbens

Mean ± SEM relative mRNA expression of (A) *Drd1*, (B) *Drd2*, (C) *Drd3*, (D) *Bdnf*, and (E) *TrkB* as determined by semi-quantitative real-time PCR, among Low LG and High LG offspring across postnatal development. All relative values were normalized to beta-actin and to the PN0 Low LG group for each transcript examined. § $p<0.1$, *$p<0.05$.

Tyrosine hydroxylase gene promoter methylation across development

To further explore the mechanism through which maternal LG shapes the development of dopaminergic pathways (as indicated by the increased number of TH-expressing cells in the VTA amongst High LG offspring), we determined whether postnatal LG was associated with variation in epigenetic programming of *Th* expression through DNA methylation of the *Th* gene promoter. Analysis of a region in the *Th* promoter containing 10 CpG sites (-269 to -94) revealed a consistent pattern of methylation across postnatal development at PN0, 6, and 21. CpG9 was 100% methylated in nearly all samples at all ages. There was a significant main effect of age on average methylation collapsed across the 10 CpG sites [$F(2,36)=18.08$, $p<0.001$; **Figure 4.4B**], such that methylation increased with age. This analysis did not reveal significant main effect of maternal LG ($p>0.8$), though at PN21 CpG site 4 was significantly more methylated among Low LG compared to High LG offspring [$t(1,8)=3.15$, $p<0.05$].

Figure 4.4

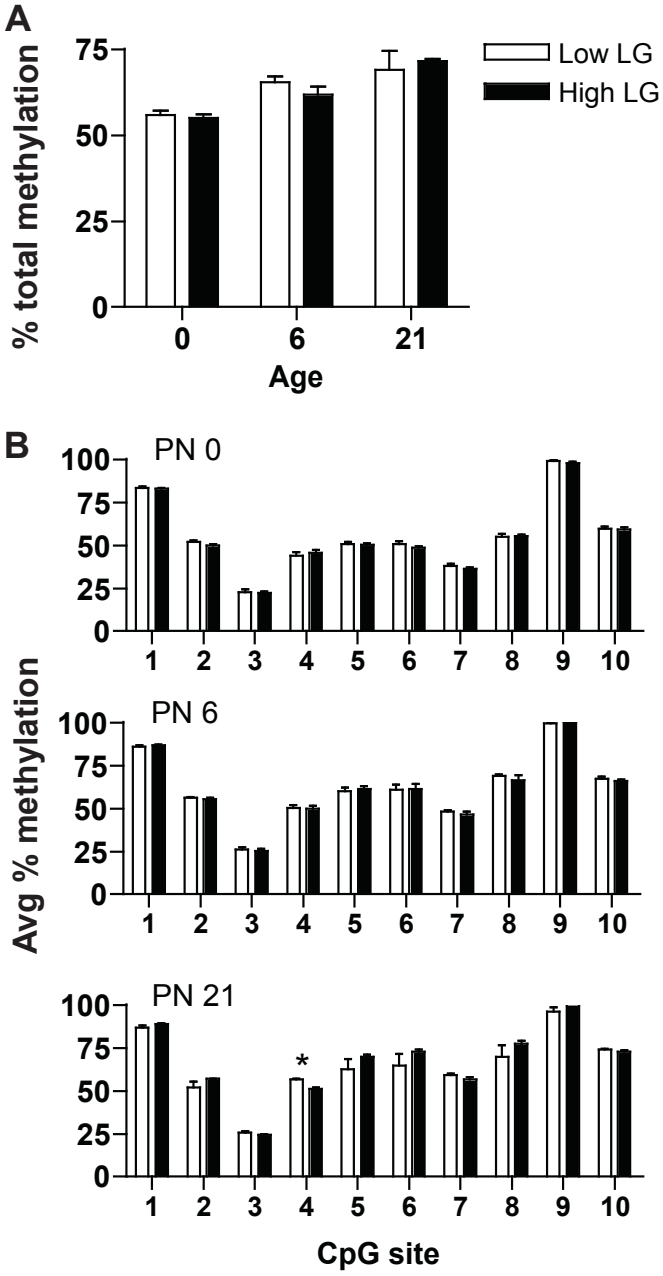

Figure 4.4 DNA methylation within the *Th* promoter of Low and High LG offspring

(A) Mean ± SEM total percent methylation from all 10 CpG sites among Low LG and High LG offspring at PN0, PN6, and PN21. (B) Mean ± SEM percent methylation at each CpG site among Low LG and High LG females at each developmental stage. *$p<0.05$.

Maternal LG affects offspring conditioned place preference for rewards

To determine the functional impact of maternal LG-associated changes in the mesolimbic dopamine system for reward/motivated behavior, Low LG and High LG offspring were tested for conditioned place preference for 1) high fat diet (HFD), and 2) social interaction and play with a sibling.

High fat diet conditioned place preference

Following conditioning, female offspring of High LG dams spent a higher percentage of time in the HFD-paired chamber compared to Low LG females [$t(1, 22)=3.14$, $p<0.01$; **Figure 4.5A**], while Low LG females spent a higher percentage of time exploring the chow-paired chamber [$t(1, 22)=3.48$, $p<0.01$]. Group differences in percent time exploring the center chamber were not observed ($p=0.75$). High LG female offspring were found to have significantly greater preference score in the HFD compared to Low LG offspring [$t(1, 22)=3.65$, $p<0.001$; **Figure 4.5B**]. This effect was confirmed by repeated measures ANOVA using 10-minute bins [main effect of maternal LG $F(1,15)=18.09$, $p<0.001$; main effect of time $F(5,15)=1.96$, $p<0.05$; interaction $F(5,15)=2.36$, $p<0.01$; **Figure 4.5C**]. There was a trend for a greater ratio of entries into the HFD / chow-associated chambers [$t(1, 22)=1.66$, $p=0.11$ **Figure 4.5D**]. Significant differences were not found among Low and High LG offspring in their latencies to enter either the HFD-paired or chow-paired chambers ($p>0.45$) or in the total distance traveled ($p>0.26$).

Figure 4.5

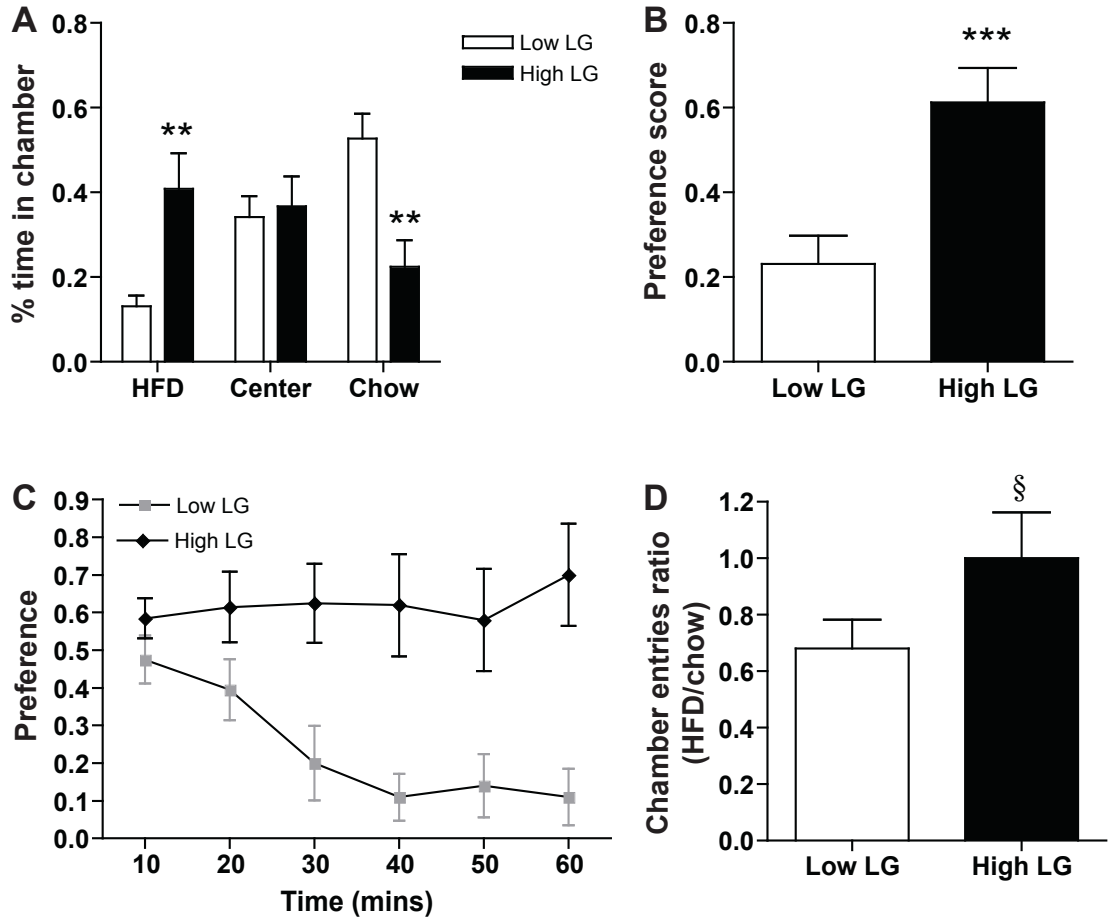

Figure 4.5 Conditioned place preference for high fat diet reward or chow control among Low and High LG juvenile offspring

(A) Percent time spent in each chamber (HFD-associated, center, and chow-associated) by Low and High LG offspring. Preference scores [(T_{HFD}) / (T_{HFD}+ T_{chow})] of Low and High LG offspring for (B) the complete 1 hour test, and (C) in ten-minute bins across the test. (D) ratio of entries into the HFD-associated chamber divided by entries into the chow-associated chamber. § $p<0.1$, *$p<0.05$, **$p<0.01$.

Social/ sibling play conditioned place preference

High LG juvenile offspring were found to spend a higher percentage of time in the toy-paired chamber compared Low LG offspring [$t(1, 18)=2.69$, $p<0.05$; **Figure 4.6A**], whereas there was only a trend for an effect on the percentage of time spent with the social-paired chamber, with Low LG offspring spending more time in this chamber [$t(1, 18)=1.85$, $p=0.08$]. There were no group differences in the percent time spent in the center chamber ($p=0.47$). In this CPP paradigm, Low LG offspring were found to travel a significantly greater distance compared to High LG offspring [$t(1, 18)=2.14$, $p<0.05$; **Figure 4.6B**], and thus distance traveled was used as a covariate in subsequent analyses. Low LG female offspring were found to have significantly greater preference score for the social chamber compared to High LG offspring [$F(1, 19)=5.49$, $p<0.05$; **Figure 4.6C**]. There was a trend for a difference in the ratio of entries into the social/toy-paired chambers [$F(1, 19)=3.92$, $p=0.06$; **Figure 4.6D]**, such that Low compared to High LG offspring had relatively more entries into the social-paired chamber. Significant group differences were also observed in the latency to enter the social-paired chamber [$F(1, 19)=6.46$, $p<0.05$; **Figure 4.6E**], but not the toy-paired chamber ($p=.97$). An additional CPP test was conducted to determine the effect of haloperidol (a D2/D1 antagonist) on preference for the social vs. toy-associated chamber. Vehicle treated females displayed the maternal LG-associated preferences observed during CPP testing on the previous day [Low LG offspring preferring the social chamber, High LG offspring preferring the toy chamber; $t(1,8)>3.48$, $p<0.01$]. However, we found no group differences in preference scores following haloperidol treatment ($p>0.53$; **Figure 4.6F**). Analysis indicated a trend for an interaction between maternal LG and drug treatment on preference score [$F(1, 19)=4.08$, $p=0.07$]. While haloperidol-treated Low LG females were not found to alter in their preference scores (compared

to vehicle treated), haloperidol-treated High LG females appeared to increase their preference for the social chamber.

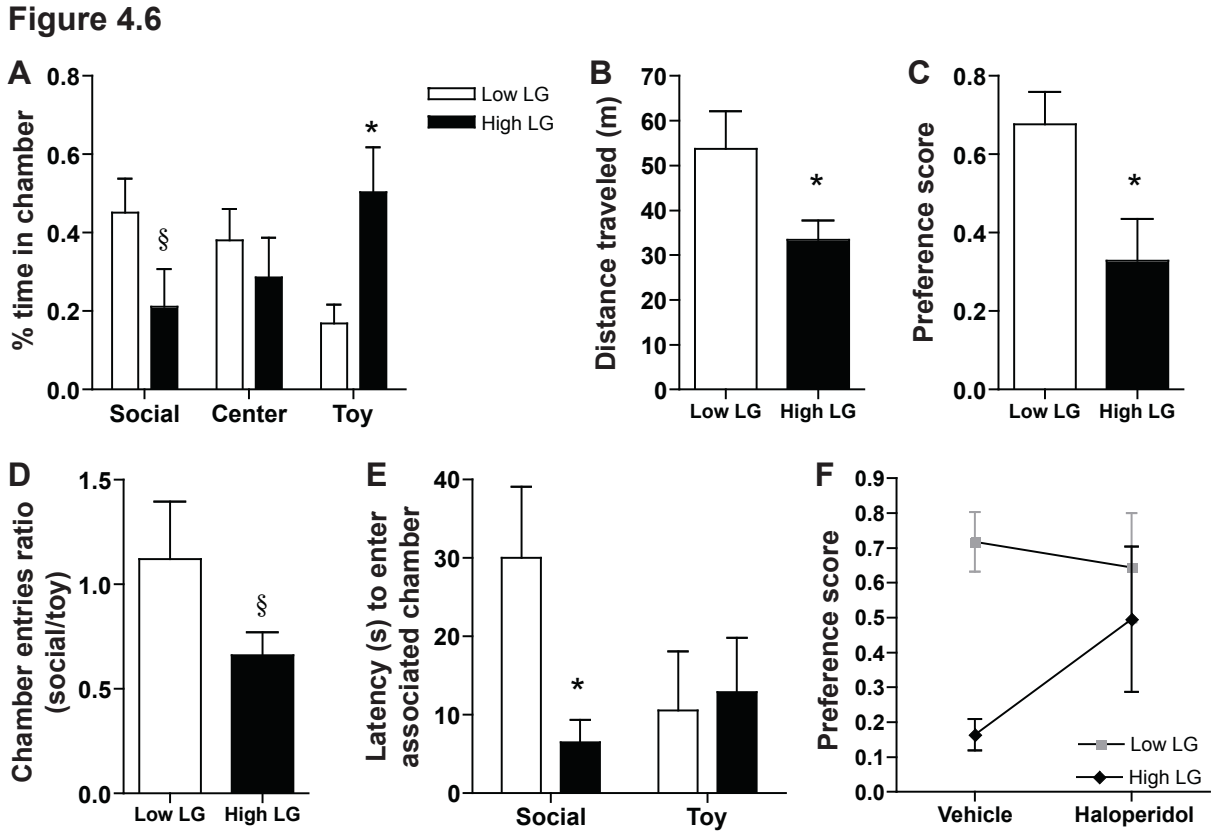

Figure 4.6 Conditioned place preference for social interaction reward or toy control

(A) Percent time spent in each chamber (sibling-associated, center, and toy-associated) by juvenile Low and High LG offspring. (B) Total distance traveled (meters) during the first preference test day. (C) Preference scores [$(T_{Sibling}) / (T_{Sibling} + T_{Toy})$] of Low and High LG offspring for the complete 1 hour test. (D) Ratio of entries into the sibling-associated chamber divided by entries into the toy-associated chamber. (E) Latency (seconds) to enter the sibling- or toy-associated chambers. (F) Preference scores of Low and High LG offspring on the second test day after administration of vehicle or haloperidol. § $p<0.1$, *$p<0.05$.

Discussion

Results of these studies demonstrate that natural variation in postnatal maternal care predicts long-lasting differences in mesolimbic dopamine system development. Our data show that maternal LG is associated with group differences in the emergence of TH-immunoreactive cells in the VTA as early as PN6, as well as group differences in postnatal expression of transcription factors necessary for midbrain dopamine neuron differentiation and maintenance. We further show that Low or High maternal LG is associated with reward-specific differences in conditioned place preferences, which may be mediated by differences in NAc dopamine D2-like receptors. Overall, these studies illustrate the influence of natural variation in maternal LG on development of dopamine pathways relevant for motivated behaviors, and thus identify a mechanism that can contribute to the transmission of maternal behavior from mothers to female offspring.

Midbrain dopamine neurons are critical for a range of motivated behaviors, including maternal behavior. Our finding of elevated levels of TH-ir cells specifically within the VTA of High LG female offspring at the end of the first postnatal week and within the PBP of the VTA in adulthood has important implications for behavior based on the connectivity and projection sites of these regions. Dopamine neurons of the PBP project to the ventral striatum, olfactory tubercle, prelimbic cortex, and infralimbic cortex (Beckstead et al., 1979; Björklund and Dunnett, 2007; Ikemoto, 2007). It has been suggested that the posterior VTA including the PBP, rather than the anterior VTA (including the PN and VTAR), mediates the rewarding effects of drugs of abuse, and that PBP connections with the NAc are particularly important for conditioned "action-arousal" behavioral responses (Ikemoto, 2007). TH-ir cells were also found

to be elevated in High vs Low LG offspring specifically in the SNL subregion of the SN. The anatomical connectivity of the SNL is also functionally relevant for motivated behaviors such as maternal behavior, as this region projects to the amygdala (Kaelber and Afifi, 1979; Woolf and Butcher, 1982; Björklund and Dunnett, 2007), though there is evidence that these projections are not dopaminergic (Loughlin and Fallon, 1983). The amygdala has been implicated in maternal behavior through is projections to the BNST and thereafter the MPOA (Fleming, 1986). Although the contribution of the amygdala to reward-directed behaviors was not examined in the current studies, activation of basolateral amygdala neurons projecting to the NAc facilitates reward seeking (Stuber et al., 2011), and lesioning the amygdala has previously been shown to reduce neophobia and enhance maternal behavior. Thus the maternal LG-induced group differences in TH-ir in the PBP and SNL may be anatomically and functionally relevant for maternal and reward-directed behavioral differences.

Important functional and neuropsychiatric consequences are associated with lower and higher dopaminergic tone in the VTA-NAc circuit. Lower dopaminergic tone is associated with anhedonia, addiction, depression, and ADHD while higher dopaminergic tone is associated with addiction, high motivational drive, altered response to drugs and stress, and positive schizophrenic symptoms (see Wise, 2008; Rodrigues et al., 2011). While baseline and stimulus-induced dopamine release were not measured in the NAc in the current study, elevated TH-ir in the VTA has been previously associated with elevated dopamine content in the NAc and increased conditioned place preference (Kostic et al., 1997; Shim et al., 2000; Vucetic et al., 2010; Liang et al., 2012). Group differences in offspring dopamine release are further suggested by enhanced maternal behavior among offspring of High compared to Low LG dams (Chapter 3; Francis et al., 1999; Champagne et al., 2003a), as well as greater dopamine release preceding and

during pup LG among High LG lactating dams (Champagne et al., 2004). These studies suggest the elevated levels of TH-ir in the VTA found here among neonatal and adult High LG offspring may be one mechanism by which the experience of maternal LG programs adult maternal behavior.

The early developing and long-lasting group differences in TH-ir observed in the VTA prompted investigation into the mechanisms contributing to these maternal LG induced alterations. One possibility we considered was that these effects were due to postnatal LG induced variation in transcription factors mediating dopamine neuron differentiation, maturation, and survival. The transcription factors Nurr1 and Cdkn1c ($p57^{kip2}$) have been shown to interact to induce cell cycle arrest and dopamine neuron differentiation (Joseph et al., 2003; Perlmann and Wallen-Mackenzie, 2004). We found *Cdkn1c* mRNA to be elevated specifically at PN6 among High compared to Low LG offspring. Cdkn1c knockout mice have increased apoptosis and delayed differentiation (Yan et al., 1997), suggesting that lower levels of *Cdkn1c* among Low LG offspring early in postnatal development could be associated with a delayed VTA development in addition to fewer dopaminergic cells. Similarly, delayed plasticity of the mesolimbic dopamine system is also observed after neonatal 6-OHDA lesions (Frohna et al., 1997). We were somewhat surprised not to find differences in *Nurr1* associated with postnatal maternal care given differences in *Cdkn1c*. Consistent with this finding, however, are reports of increased TH-ir cells in culture in response to prolonged membrane depolarization without altered Nurr1-ir or *Nurr1* mRNA levels (He et al., 2011), which was found to be due to an increased proportion of Nurr1-expressing cells committing to a TH+ phenotype after depolarization. In both cases this may be due to differences in Nurr1-Cdkn1c interactions,

leading to altered binding and activation at the *Th* promoter (Lenartowski and Goc, 2011), but further studies are needed to assess this possibility among Low and High LG offspring.

At later time points (PN21, PN66/ adulthood) when dopamine neuron differentiation is presumed to be complete and the number of dopamine neurons is sensitive to transcription factors regulating maturation and survival, we found *Lmx1b* to be elevated among High LG compared to Low LG offspring. Lmx1b and Pitx3 are transcription factors implicated in midbrain dopamine neuron maintenance and survival. Previous experiments showed *Lmx1b* null mice were initially able to generate TH-expressing neurons, but the neurons did not survive and *Th* was undetectable after E16 (Smidt et al., 2000). Pitx3, widely expressed in development but restricted in adulthood exclusively in the VTA and SN, is also diminished in *Lmx1b* mutant mice (Smidt et al., 1997; Smidt, 2004). *Lmx1b* and *Pitx3* are thought to represent a second pathway for dopamine neurons differentiation and maintenance, independent of *Nurr1* and *Cdkn1c*, because *Lmx1b* is normally expressed in *Nurr1* mutant mice (Smidt et al., 2000; Prakash and Wurst, 2006). Our results are consistent with previous findings showing decreased levels of *Lmx1b* mRNA and unaltered *Pitx3* mRNA in response to methamphetamine challenge in adult rats (Krasnova et al., 2011). The age-specific maternal LG group differences observed in these transcription factors suggest that maternal LG predicts both dopamine neuron production and survival, and thus provides a mechanism for organizational, long-lasting effects on midbrain dopamine neuron development.

A second possible explanation for the long-term group differences observed here in TH-ir, was altered levels of *Th* gene expression within the TH-ir cell population. The population of cells expressing TH is not necessarily directly associated with the level of *Th* mRNA. For example, a previous study showed that in VTA sections probed for *Th* mRNA by *in situ*

hybridization and simultaneously immunostained for TH, most but not every immunostained cell showed high levels of *Th* mRNA (Seroogy et al., 1989). Likewise post-transcriptional and post-translational modifications are believed to affect translation or stability of TH protein independent of the level of transcription (Lenartowski and Goc, 2011), as seen by increased TH protein but not mRNA after induction by forskolin and cAMP (Tank et al., 2008). Conversely, induction of *Th* mRNA does not always lead to induction of TH protein (Baruchin et al., 1990; Nankova et al., 1994; Piech-Dumas et al., 1999). We therefore examined *Th* promoter methylation in the ventral midbrain, as promoter methylation is thought to be stable across the lifespan (Bird, 2002; Schaefer et al., 2007; Ooi and Bestor, 2008a; Sharma et al., 2010) and could represent another mechanism for long-term changes in TH-ir. The promoter region examined in the current study was previously associated with chromatin remodeling and moderate CpG methylation differences (He et al., 2011) and contains regulatory sequences for AP-1, SP-1, and HRE, and is just upstream of a CRE/CaRE element and transcription start site (Lenartowski and Goc, 2011). However, while methylation across the 10 CpG sites examined was found to increase across postnatal development, maternal care was not associated with significant differences in *Th* promoter methylation. Further research is needed to understand whether more dynamic epigenetic regulation of *Th* at the level of chromatin remodeling takes place across postnatal development and in association with maternal LG. Together these findings promote the idea that postnatal maternal LG has long-term effects on mesolimbic dopamine system development via alterations at the level of dopamine neuron-promoting transcription factors rather than *Th* promoter methylation.

To explore whether the differences discovered in Low and High LG offspring mesolimbic dopamine system development were relevant for reward-directed behaviors, we

assessed animals' conditioned place preference behavior for two natural rewards: high fat diet and social interaction/ play. HFD is a palatable natural reward and its preference is mediated by NAc dopamine receptors (Baker et al., 2001; Zhang et al., 2003; Teegarden et al., 2009). Social play is likewise regulated by the mesolimbic dopamine system and previously found to be naturally rewarding among juveniles (Einon et al., 1981; Thor and Holloway, 1984; Calcagnetti and Schechter, 1992; Vanderschuren et al., 1997; Trezza et al., 2010; El Rawas et al.; Peartree et al., 2012). Social play increases around PN18 and reaches a peak around PN30-40 during the juvenile period (Panksepp, 1981; Spear and Brake, 1983), at the age when females were tested for social interaction/play conditioned place preference. Thus, in the current studies, HFD and social play were considered to be the rewarding stimuli. Consistent with the current finding of decreased HFD place preference among Low LG offspring, an unstable maternal environment created by repeated cross-fostering from PN1-4 similarly decreased preference for palatable foods and saccharine intake (Ventura et al., 2012). While all animals were exposed to HFD once prior to testing, it is possible that Low LG offspring were inhibited by the relative novelty of the HFD compared to chow, consistent with behavioral inhibition exhibited by Low LG offspring in open novel environments (Francis et al., 1999). However, Low LG offspring had greater place preference for sibling interaction compared to High LG offspring, indicating that Low LG offspring are not simply anhedonic, as might be suggested by lower VTA TH-ir (Wise, 2008). Increased place preference for sibling interaction is consistent with a theory of "play compensation" among maternally deprived offspring that could make social stimuli in the juvenile period particularly salient among Low LG but not High LG offspring (Parent and Meaney, 2008). Monkeys (Goy and Wallen, 1979; Bernstein and Dobrofsky, 1981; Wallen, 1996) and rats (Panksepp, 1981; Beatty et al., 1982) that were maternally separated or socially

isolated early in life significantly increase play behaviors in the juvenile period relative to control animals, and this increase has been theorized to compensate for lack of maternal, social, or tactile stimulation earlier in development (Bernstein and Dobrofsky, 1981). Additional support for this theory comes from previous findings of increased home-cage play behaviors among offspring that received lower levels of LG (Birke and Sadler, 1987; Moore and Power, 1992; Parent and Meaney, 2008; Parent et al., 2012). However it should be noted that other studies have found a positive relationship between LG received and play behaviors in male offspring after brief social isolation (Van Hasselt et al., 2012). These results suggest that the maternal LG-induced variation in dopaminergic cells of the VTA is relevant for stimulus-specific motivated behaviors. Variation in other brain pathways that may contribute to the stimulus-specific nature of these preferences has yet to be elucidated.

We also show that variation in NAc dopamine receptor levels induced by variation in maternal LG is a likely mediator of these behavioral preferences. Consistent with our finding of elevated mRNA levels of dopamine receptors D1, D2, and D3 in the NAc of High LG offspring at PN21 (proximate to the age of behavioral testing), the mixed D1/D2-like antagonist haloperidol reduced place preference among High to a greater extent than among Low LG offspring. Likewise, haloperidol has previously been shown to inhibit pup retrieval and LG among lactating dams (Stern and Taylor, 1991), and D1 activation was found to enhance maternal sensitization and pup retrieval, suggesting that variation in dopamine receptor levels among Low and High LG offspring may be functionally relevant for maternal behavior.

This is one of the first studies to show that natural variation in the quality of the early maternal environment contributes to variation in offspring VTA and NAc development and motivated behavior for natural rewards, which may be adaptive under different environmental

conditions. Offspring reared in unstable maternal conditions were previously found to have blunted dopamine release in the medial prefrontal cortex (mPFC) in rewarding and aversive conditions (access to chocolate, or LiCl injection, respectively), and enhanced mPFC norepinephrine release in response to aversive conditions (Ventura et al., 2012). It was suggested that an unstable maternal environment impairs pleasure-seeking and enhances sensitivity to negative events, which could be adaptive in stressful environments. Consistent with the theory of enhanced sensitivity for stressful events, Low LG male offspring were previously shown to have elevated dopamine release in the mPFC after tail-pinch stress (Zhang, 2005), as well as enhanced fear-conditioned learning compared to High LG offspring (Champagne et al., 2008; Bagot et al., 2009). Thus, in a stressful environment it may be adaptive to display enhanced sensitivity to social stimuli (that could provide valuable information about the environment, or could possibly be stressful if accompanied by challenge of hierarchical social standings), while in less stressful environments it could be adaptive for palatable food, pups, and other stimuli to be sufficiently salient and motivating.

Chapter 5: Postnatal over-expression of estrogen receptor-alpha reverses the effects of low maternal care in female offspring and modifies TH levels in the ventral tegmental area

Introduction

Maternal care is dependent upon estrogen receptor-alpha (ERα) and oxytocin receptor (OTR) signaling in the medial preoptic area (MPOA) of the hypothalamus, as well as dopamine signaling in the mesolimbic dopamine pathway, involving projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (see Chapter 1; Lonstein and Morrell, 2006). Variations in these two brain systems have also been linked with variations in rat maternal licking and grooming (LG). High compared to Low LG lactating dams were previously found to have elevated levels of OTR binding and ERα mRNA in the MPOA, as well as increased dopamine release in the NAc during pup LG (Champagne et al., 2003b; Champagne et al., 2004). Although the hypothalamic neuroendocrine system and the mesolimbic dopamine system are typically studied separately, they are anatomically connected and both implicated in maternal behavior. For example, pups were only able to elicit dopamine release in the NAc of postpartum dams or females that had been hormonally primed (Afonso et al., 2009), and pharmacological activation of D1 receptors in the NAc was only able to enhance maternal sensitization and pup retrieval in pregnancy-terminated (hormonally primed) females (Stolzenberg et al., 2007). The importance of the connectivity of these two systems for maternal behavior is further evidenced

by a previous study that found High LG dams to have more oxytocin neurons projecting from the MPOA to the VTA relative to Low LG dams (Shahrokh et al., 2010). Infusion of oxytocin directly into the VTA induced elevated dopamine release in the NAc compared to saline (Shahrokh et al., 2010), indicating the functional relevance of the variation in oxytocin projections for maternal behavior.

In the previous chapters, the impact of maternal LG on hypothalamic and dopaminergic systems in offspring has been demonstrated. Female offspring reared by High compared to Low LG dams have elevated levels of ERα-immunoreactive (-ir) cells in the MPOA (Chapter 3) and tyrosine hydroxylase (TH-ir; as a proxy for dopamine neurons) cells in the VTA at PN6 (Chapter 4). High LG female offspring also had a faster onset of maternal behavior compared to Low LG offspring (Chapter 3). The early postnatal emergence of differences in ERα-ir and TH-ir in Low and High LG offspring suggest that these pathways develop independently. However, there is also evidence to suggest estrogen receptor signaling directly affects midbrain dopamine neurons. Cultured dopamine neurons treated with estrogen increased neurite growth and TH mRNA (Raab et al., 1995). Treatment of ovariectomized female rats with estrogen increased the probability of VTA dopamine neuron firing (Sakamoto et al., 1993). Effects of estrogen on dopamine neurons are likely mediated through ERα. TH-ir cells in the VTA were reduced by ovariectomy and rescued by treatment with estradiol or an ERα agonist, but not an ERß agonist (Johnson et al., 2010). ERα but not ERß knockout mice also had fewer TH-ir cells in the VTA compared to controls (Johnson et al., 2010). Estrogen also induces dopamine efflux in culture, which is blocked by knockdown of ERα but not ERß (Alvea et al., 2009). ERα was transiently found in the VTA from embryonic day 17 to postnatal day 20 in rodents, indicating that the VTA may be

particularly sensitive to ERα during early postnatal development (Raab et al., 1995; Raab et al., 1999; Beyer et al., 2003).

These previous findings raise two important questions: 1) Are elevated levels of ERα during the post-natal period sufficient to drive maternal behavior? and 2) Does elevated neonatal hypothalamic ERα drive changes within the mesolimbic dopamine system? In order to answer these questions, we took advantage of the variation in ERα-ir, TH-ir, and maternal sensitization behavior induced by Low and High maternal LG. This variation allowed us to test whether ERα over-expression in Low LG offspring that normally have low levels of ERα could elevate the levels of maternal behavior and TH-ir to that of High LG offspring. We were successfully able to manipulate ERα levels in the MPOA of offspring and show that maternal LG or ERα affect both onset of maternal behavior and the levels of TH-expressing cells in the VTA.

Results

Animals included in analysis

In order to elucidate whether postnatal increases in ERα expression elevate the level of maternal behavior and dopaminergic cells in the ventral midbrain of Low and High LG offspring, adenovirus was used to deliver GFP control or ESR1 to the MPOA of neonates that underwent maternal sensitization testing after weaning. **Figure 2.5** illustrates (**A**) the timeline of maternal observations, neonatal brain injections, and maternal sensitization testing and (**B**) the composition of all animals injected and behaviorally tested. Of the 48 animals tested, 9 came from Mid LG litters. Due to uncertainty as to the number of donor pups available for maternal sensitization testing, pups from Low and High LG litters were prioritized and thus Mid LG females began testing with a slight age lag (Low and High LG females began at age PN23-26,

Mid LG females began at PN28-29). Linear regression with LG frequency and age at start of testing as continuous independent variables revealed a significant effect of start age on latency to maternal sensitization behavior [$F(2, 44)=4.03$, $p<0.05$], due primarily to longer latencies among the Mid LG females that began testing 2-3 days later. These animals also had lower levels of ERα-immunoreactive cells which may account for the longer latencies, but because the cause of these outcomes was ambiguous, they were removed from further analysis. Six animals (3 Low LG, 3 High LG) also had low ESR1 staining when the recorded injection was GFP, indicating potential contamination, and were excluded from further analysis. Thus, the numbers of animals used for analyses in each group are: 7 Low LG and 6 High LG control/ Ad-GFP animals, and 9 Low LG and 11 High LG Ad-ESR1 animals.

ESR1 over-expression

At the level of the MPOA, ESR1-ir was restricted to 2 mm on either side of the third ventricle, indicating that the virus was taken up by the tissue surrounding the injection site but that spread in the immediate vicinity was moderate and within previously reported range (Rahim et al., 2011). Average MPOA staining was modest with a mean of 36 cells (per hemisphere, averaged across sections) and a range from 8 to 104 mean cells per MPOA hemisphere detected. There was no significant difference in ESR1-ir cells counted among Low and High LG females ($p=0.28$; **Figure 5.1A**, **C**), nor was ESR1 correlated with maternal sensitization latency ($p=0.25$). MPOA staining was observed bilaterally in most cases. Surprisingly, the pattern of ESR1 staining was atypical compared to endogenous ERα staining. ERα-ir is commonly observed within the cell nucleus with occasional reports of diffuse soma staining. However, ESR1-ir was seen in puncta, presumed to localize to the cell body, as well as along tracts. Although ESR1

was under the control of a different promoter, there was no reason to believe that trafficking of the protein should be different from endogenous ERα.

ESR1 staining was also detected in regions outside of the MPOA, including posterior regions of the hypothalamus, the lateral edge of the ventricles, and within the cortex (**Figure 5.1B, D**). Cortical staining is likely due to virus being absorbed by dividing and cortically migrating cells of the subventricular zone. Cortical staining was observed in somatosensory and cingulate cortices and to a lesser degree in the piriform cortex. The pattern of puncta and tracts were also present in these regions. There was no significant difference in the average cortical ESR1-ir cells counted among Low and High LG females (p=0.13), nor was there a correlation between cortical ESR1-ir and maternal sensitization latency (p=0.73). Staining was observed in thalamic and amygdalar regions in three cases, which were excluded due to the low occurrence of this pattern and therefore the inability to statistically link it to behavior. No strong center of staining was observed, likely due to the growth and migration of neurons during early postnatal development. Together, these findings show that injection of Ad-ESR1 into the brains of neonates did result in expression of ESR1 in the MPOA and medial ventral hypothalamus, as well as cortical regions, and that expression of ESR1 was not statistically different among Low and High LG offspring.

Figure 5.1

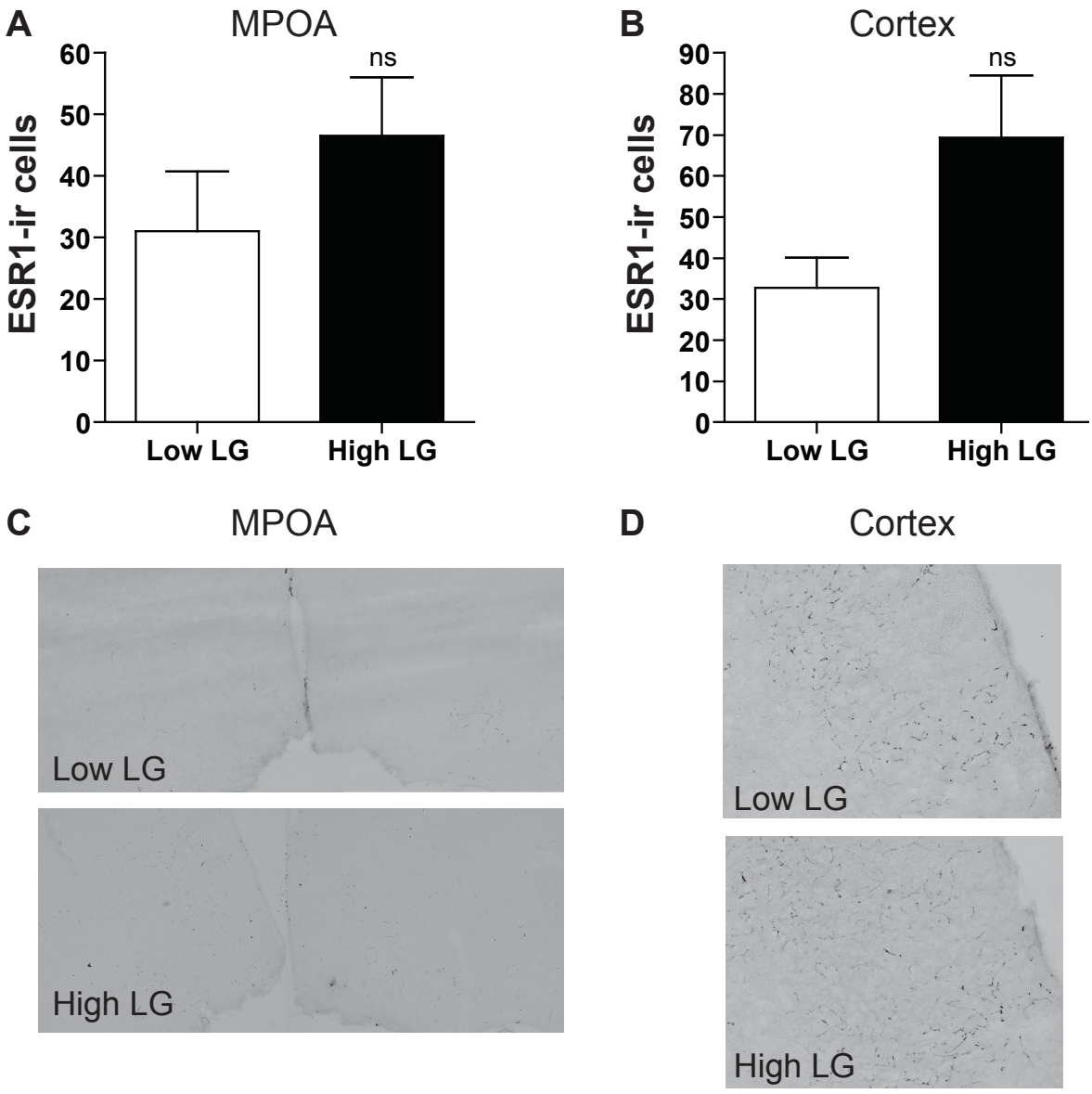

Figure 5.1 ESR1-ir cells are detected in the brains of juveniles

ESR1-ir was detected in the (A, C) MPOA and (B, D) cortex of females that received neonatal injections of Ad-ESR1. There was no statistical difference in the extent of expression in either of these regions among Low and High LG offspring. ERS1-ir was detected bilaterally in the (C) MPOA and (D) cortex. A pattern of puncta and tracts was observed wherever immunoreactivity was found.

ERα immunoreactivity in the MPOA

Maternal LG has been linked to variation in levels of ERα mRNA and immunoreactivity within the MPOA of female offspring (Chapter 3; Champagne et al., 2003b; Champagne et al., 2006). To investigate this difference in control animals and determine the effect of Ad-ESR1, the level of ERα-ir cells in the MPOA was analyzed among all animals. Two-way ANOVA indicated a main effect of maternal LG group [$F(1,29)=5.64$, $p<0.05$)] and an interaction between maternal LG group and Ad-ESR1 injection [$F(1,29)=5.66$, $p<0.05$, **Figure 5.2A-C**]. Additional comparisons revealed a significant difference in ERα-ir cells among Low and High LG control females [$t(1, 10)=5.90$, $p<0.001$], such that there were elevated levels of ERα-ir cells among High compared to Low LG control females. No significant difference in ERα-ir cells was found among Low and High LG ESR1-injected females ($p>0.99$). Among control and ESR1 Low LG females, there was a significant difference in ERα-ir cells [$t(1, 13)=3.28$, $p<0.01$], which was not detected among control and ESR1 High LG females ($p>0.47$). Additionally, there was a significant correlation between maternal LG frequency and ERα-ir cells in the MPOA among control [$R(16)=0.83$, $p<0.001$; **Figure 5.2D**] but not ESR1-injected females ($p=0.94$). These results indicate that High maternal LG or over-expression of ESR1 is sufficient to increase levels of ERα-ir cells in the MPOA of females, but that these effects are not additive.

Figure 5.2

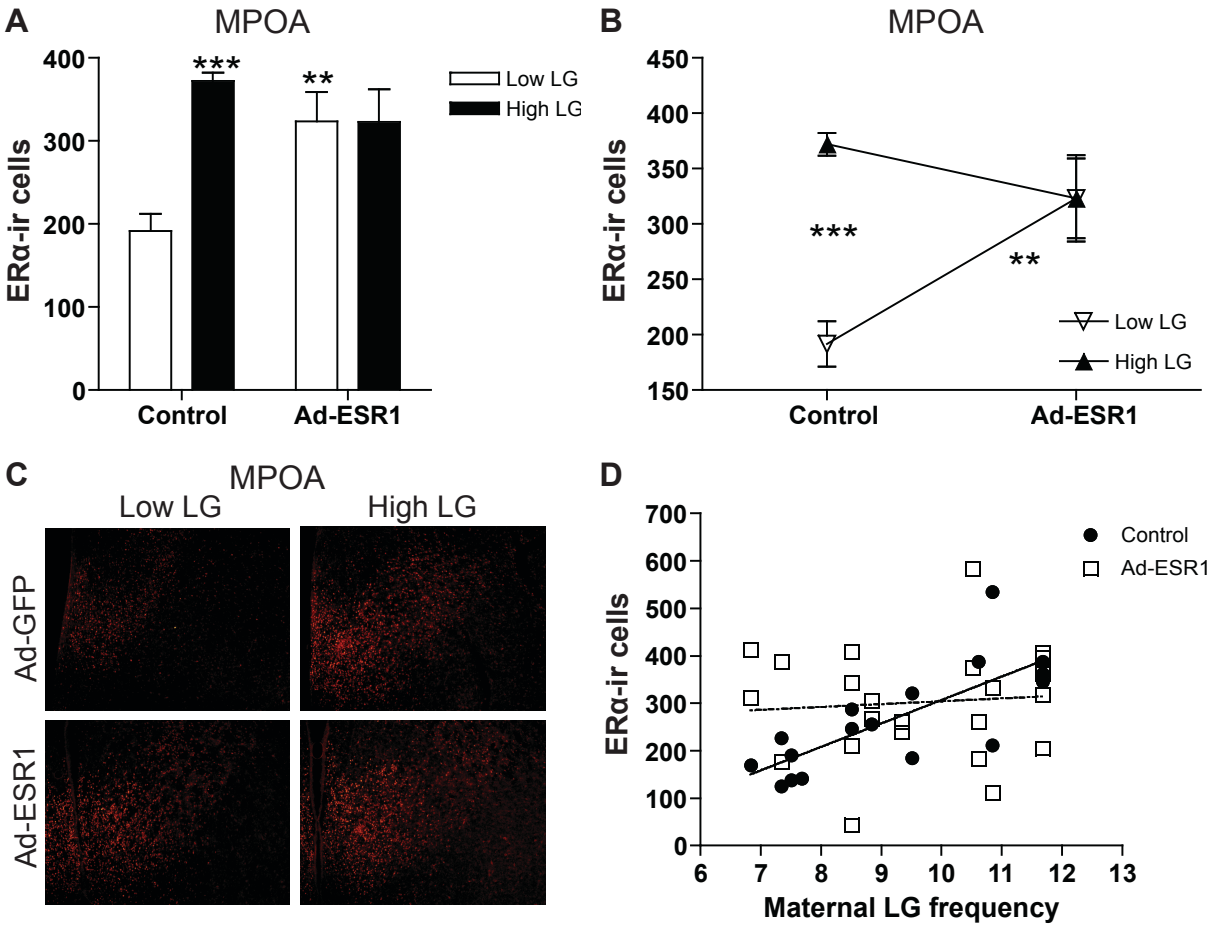

Figure 5.2 ERα-ir cells in the MPOA of control and Ad-ESR1 Low and High LG offspring

(A, B) Mean ± SEM cells counted expressing ERα protein in the MPOA of control and Ad-ESR1 Low and High LG offspring, as seen in the representative images in (C). The effect of Ad-ESR1 on Low LG offspring was highlighted in (B). $^{**}p<0.01$, for the comparison between Ad-ESR1 and control; $^{***}p<0.001$ for the comparison between control Low LG and High LG. (C) Representative images of ERα-ir in the MPOA show one hemisphere, with the third ventricle to the left and MPOA to the right. (D) Correlations between maternal LG frequency and ERα-ir in the MPOA were significant ($p<0.05$) only among Low LG offspring.

Maternal sensitization behavior

The latency for initially pup-avoidant virgin females to sensitize to neonatal pups has been linked to the female's own maternal LG frequency upon mating and parturition, and with estrogen-inducible oxytocin receptor levels (Champagne et al., 2001; Chapter 3). We sought to understand whether Low or High maternal LG levels and developmental over-expression of ESR1 predicted juvenile maternal sensitization latency. Analysis revealed High LG offspring to have significantly shorter latencies to maternal sensitization compared to control Low LG females [$t(1, 10)=2.51$, $p<0.05$; **Figure 5.3A, B**]. No statistical difference was found in the latencies of Low and High LG females that received ESR1. Among Low LG females, those that received ESR1 had significantly shorter latencies to maternal sensitization compared to controls [$t(1, 14)=2.39$, $p<0.05$; **Figure 5.3A, B**], a difference that was not detected among High LG females ($p>0.47$). We also examined correlations between ERα-ir or TH-ir and behavior. The number of ERα-ir cells in the MPOA were significantly correlated with latency to maternal sensitization behavior [$R(29)=0.37$, $p<0.05$; **Figure 5.3C**]. These results indicate that High maternal LG or over-expression of ESR1 in the brain is sufficient to decrease maternal sensitization latency. However, it is not possible to determine if these effects are additive, due to a potential floor effect in the behavioral latency.

Figure 5.3

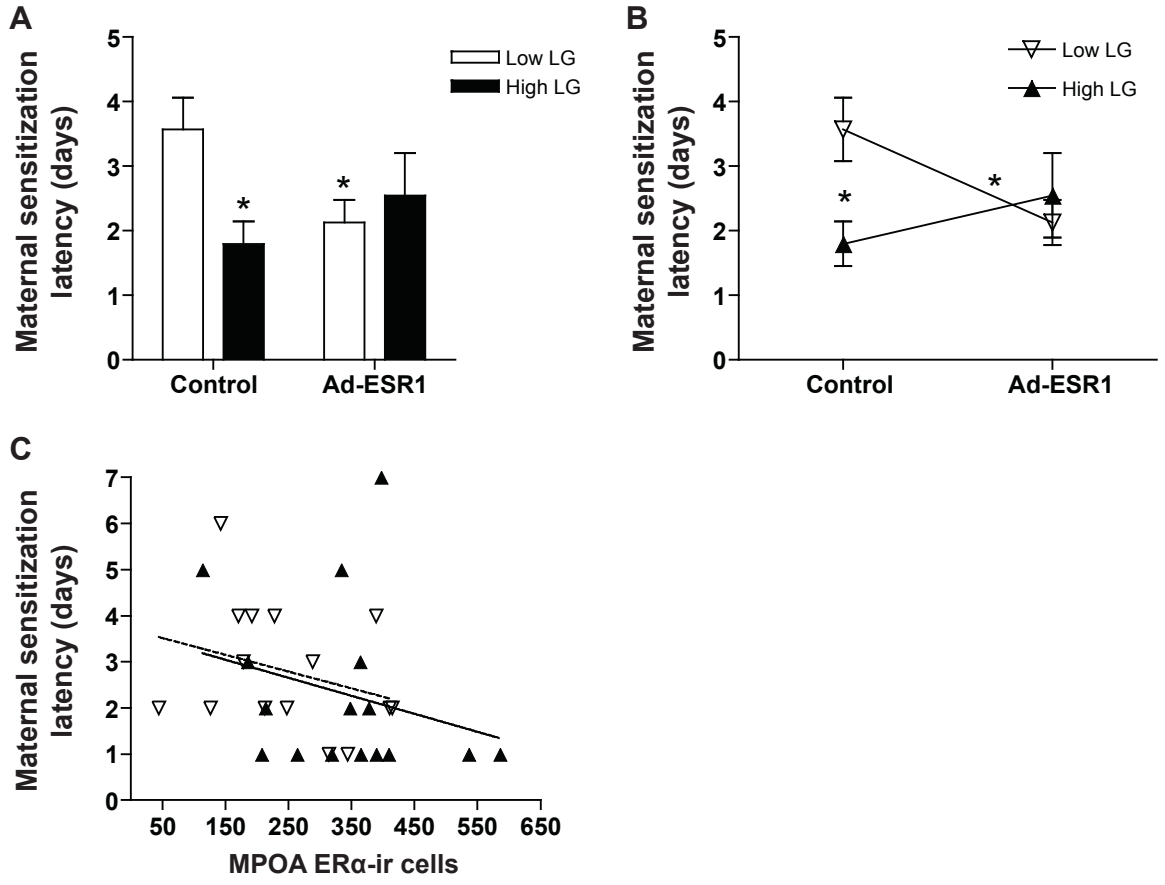

Figure 5.3 Maternal sensitization latencies of control and Ad-ESR1 Low and High LG offspring

(A, B) Mean ± SEM latency (days) of juvenile females to sensitize to neonatal pups and show full maternal behavior within the 1-hour test period, as defined by grouping all 3 donor pups to a nest, crouching over pups, and licking/ grooming pups. The effect of Ad-ESR1 on Low LG offspring latency was highlighted in (B). Comparisons were made between Low and High LG offspring (within control or Ad-ESR1 treatment groups) or between treatment groups (among Low LG or High LG offspring); *$p<0.01$ for the comparisons between control Low and High LG offspring and Low LG control and Ad-ESR1 offspring. (C) Correlation between ERα-ir cells in the MPOA with maternal sensitization latency among Low and High LG offspring.

TH immunoreactivity in the ventral midbrain

Elevated levels of TH-ir cells in the VTA of High compared to Low LG female were previously found at the end of the first postnatal week and in adulthood (Chapter 4). An outstanding question was whether maternal LG induced variation observed in MPOA ERα cells and VTA dopamine cells separately, or whether the differences in VTA dopamine neurons might be downstream of developmental differences in MPOA ERα levels. TH immunoreactivity in the VTA and SN was therefore analyzed in all animals. Two-way ANOVA indicated a significant main effect of maternal LG group [$F(1,32)=7.19$, $p<0.05$; **Figure 5.4A,D**] but not a significant effect of virus ($p>0.16$) nor an interaction ($p>0.64$) on TH-ir cells counted in the whole VTA. This was primarily driven by differences in the PBP [main effect of maternal LG, $F(1,32)=7.26$, $p<0.05$; **Figure 5.4B**], as there was not a significant main effect of maternal care found for any other VTA nucleus. Further analysis indicated a significant difference among Low and High LG control animals in TH-ir cells in the PBP [$t(1, 12)=3.62$, $p<0.01$] and entire VTA [$t(1, 12)=3.47$, $p<0.01$], such that more TH-ir cells were found among High LG females. Among control and ESR1 Low LG females, there was a trend for differences in TH-ir cells in the PBP [$t(1, 14)=2.00$, $p=0.065$], such that Low LG females that received Ad-ESR1 had more TH-ir cells compared to controls. These differences were not detected among High LG females ($p>0.57$). There was also a positive linear correlation between ERα-ir cells in the MPOA and TH-ir cells in the PBP [$R(31)=0.34$, $p<0.05$; **Figure 5.4C**]. There was not a significant effect of maternal LG or ESR1 over-expression on TH-ir cells in the SN or any of its nuclei. Finally, there was a trend for the number of TH-ir cells in the PBP [$R(31)=0.33$, $p=0.07$; **Figure 5.4E**] to be correlated with latency to maternal sensitization behavior. Together, these findings suggest that there is an effect of maternal LG and of ERα in the MPOA on dopamine neurons in the VTA.

Figure 5.4

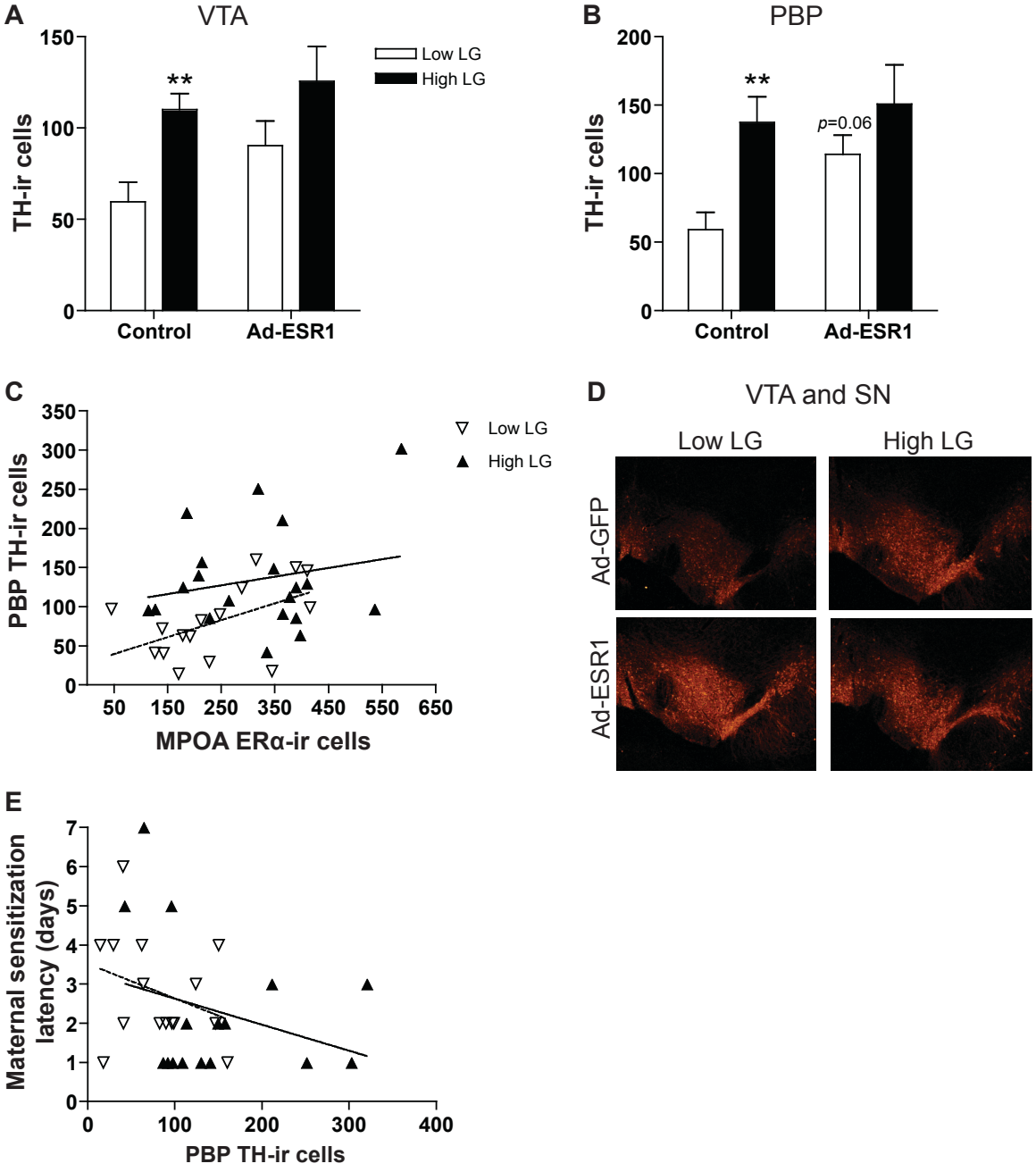

Figure 5.4 TH-ir cells in the ventral midbrain of control and Ad-ESR1 Low and High LG offspring

Mean ± SEM cells counted expressing TH in the (A) entire VTA and (B) parabrachial pigmentosa nucleus of the VTA. A significant difference (**$p<0.01$) was found in both regions for the comparison between control Low LG and High LG, while a trend was found ($p=0.06$) for the comparison between control and Ad-ESR1 Low LG females. (C) Correlations between maternal LG frequency and TH-ir in the PBP were significant ($p<0.05$) only among Low LG offspring. (D) Representative images of TH-ir in the ventral midbrain. Images show one hemisphere, with the VTA to the left and SN to the right. (E) Correlation between TH-ir cells in the PBP with maternal sensitization latency among Low and High LG offspring

Discussion

Here, we provide evidence that over-expression of ERα in the MPOA of neonatal Low LG offspring decreased latencies to maternal behavior. Our findings also show that High maternal LG is associated with greater numbers of TH-ir cells in the VTA of offspring, and that among Low LG offspring a relationship exists in the levels of ERα-ir and TH-ir cells. Overall, these findings support the conclusion that hypothalamic ERα is critical for the emergence of variation in maternal LG and indicate that manipulation of hypothalamic ERα may induce alterations within the mesolimbic dopamine system.

We show that virally-mediated expression of ESR1 in the neonatal rat hypothalamus results in lasting changes in ESR1 expression through the juvenile period. The extent of adenovirus spread was comparable to a recent exploratory study in mice. Rahim and colleagues (2011) administered 5µl intracranial Ad5-GFP to neonates on PN1 and found expression at one month around the ventricles and site of injection, comparable with the spread of virus we observed. Spread of viral expression was also previously found to be more restricted when neonates were injected compared to pups injected *in utero* (Rahim et al., 2011). Both the ventricular and cortical expression (found here and by Rahim et al., 2011), and the greater spread found after prenatal injection (Rahim et al., 2011), are consistent with cortical migration of neurons from the subventriclar zone (SVZ) in the developing brain (Doetsch and Alvarez-Buylla, 1996; Huttner and Brand, 1997). While ERα is found in the cortex, its expression is reduced significantly in the second postnatal week (Prewitt and Wilson, 2007) and cortical over-expression was a non-specific target in the current study.

This study is among the first to explore the effects of neonatal brain-targeted gene over-expression, although viral or conditional gene silencing and over-expression in culture have been studied more extensively. Demeneix and colleages (Guissouma et al., 1998; Becker et al., 2001) transfected neonatal mice with plasmids expressing thyrotropin-releasing hormone or NCoR (nuclear corepressor) and SMRT (silencing mediator of retinoic and thyroid hormone receptors) in the hypothalamus and revealed altered thyroid receptor regulation 18 hours after transfection. Jang and Goldman (2011) over-expressed the transcription factor Pax6 in neonatal mice within the SVZ, and after four days Olig2 was found to be down-regulated (Jang and Goldman, 2011). These studies show successful neonatal gene over-expression but the effects were not long-lasting or the mice not allowed to survive long enough to evaluate potential alterations at the level of behavior. To our knowledge, this is the first study to explore the effect of neonatal brain-targeted gene over-expression in the context of early environmental experience and behavior.

Our results demonstrate for the first time that increased levels of ERα in the MPOA of Low LG offspring are sufficient to facilitate maternal behaviors, as indicated by a decreased latency for onset of maternal behavior. A relationship between levels of MPOA ERα and maternal care was implicated by previous research that showed female rats reared by High compared to Low LG dams have higher levels of ERα mRNA in the MPOA, shorter latencies to maternal sensitization, and increased frequencies of LG towards their own pups (Chapter 3; (Champagne et al., 2001; Champagne et al., 2003b; Champagne et al., 2006), and by the finding that suppression of ERα in the MPOA of mice inhibited maternal care, including pup retrieval (Ribeiro et al., 2012). Our findings provide evidence for a direct relationship between ERα in the MPOA and maternal behavior, building on these previous correlational findings. It should be

noted that over-expression of ERα in the MPOA was induced during early neonatal development, but it remains to be tested whether adult over-expression of ERα would have similar effects. Together with these studies, our results suggest that the level of maternal LG experienced mediates offspring maternal behavior via developmental influence on MPOA ERα.

As previously described, the mesolimbic dopamine system is also critically involved in maternal behaviors, is hormonally sensitive, and is a direct neuroanatomical target of estrogen-sensitive oxytocin neurons of the MPOA (reviewed in Chapter 1). Compared to Low LG dams, High LG dams have been shown to have increased dopamine release and dopamine receptor binding in the nucleus accumbens (Champagne et al., 2004), but it was not clear whether this variation was directly due to experience of maternal care in infancy, stimulation by the hypothalamic neuroendocrine system, or both. Here we provide evidence that increased levels of maternal LG are sufficient to induce increased numbers of TH-ir cells in the VTA, particularly within the PBP nucleus. Our finding that, among females reared by Low LG dams, increasing levels of ERα-ir cells were significantly correlated with TH-ir cells in the VTA, suggests that the effects of LG on TH-ir cells in the VTA may be mediated by MPOA ERα. Because ERα and aromatase are expressed in the VTA in the first postnatal week, but not later in life, (Raab et al., 1995; Raab et al., 1999; Kuppers et al., 2001), these effects may be due to an organizational effect of developmentally increased MPOA ERα rather than continually-elevated expression. Alternatively, ERα-ir neurons of the MPOA may continue to affect dopamine neurons of the VTA indirectly through stimulation by oxytocin and glutamatergic afferents (Morrell et al., 1984; Geisler et al., 2007; Shahrokh et al., 2010). This possibility could be tested by perinatal over-expression and adult knock-down of ERα in the MPOA. An organizational effect would be confirmed by elevated TH-ir levels in adulthood after temporarily over-expressing ERα during

development. While these studies suggest that levels of maternal LG or MPOA ERα are associated with levels of dopamine neurons of the PBP, it remains unclear whether maternal LG acts upon the VTA directly (for example by influencing the levels of transcription factors important for VTA development, Chapter 4), indirectly via hypothalamic ERα, or both. In order to fully distinguish these possibilities, the effects of suppressing ERα in the MPOA of High LG female offspring on TH-ir in the VTA need to be examined. In addition, while our findings imply that elevated MPOA ERα expression is sufficient to facilitate onset of maternal behaviors, it has yet to be determined whether increasing the number of dopamine neurons in the VTA during development would have similar effects on behavior.

We hypothesize that because Ad-ESR1 injection failed to significantly elevate ERα-ir and TH-ir in High LG offspring, there may be a ceiling effect for the level of ERα-ir cells observed in the MPOA. This mirrors our finding that maternal sensitization was not enhanced in the High LG over-expression group. Together these findings highlight methodological limitations of the present study. Our present aim was to test the effects of increasing ERα early in postnatal development, and thus adenovirus was preferable to other vectors that can take several weeks to reach maximal expression. However, adenovirus expression can diminish over time because it does not incorporate into the host genome, and therefore maternal sensitization was the best measure of maternal care that could be examined within the relevant period of expression. Maternal sensitization among adult rats has been correlated with LG frequency upon mating and parturition (Champagne et al., 2001), and thus juvenile onset of maternal behavior is an appropriate proxy for adult maternal LG behavior. However, future studies should seek to determine the effects of ERα over-expression on individual differences in LG frequency itself, which may escape ceiling effects.

Together our results describe a model (see **Figure 5.5**) in which both elevated maternal LG or MPOA ERα facilitate onset of maternal behaviors. In addition, elevated levels of maternal LG or developmental ERα are associated with dopamine neurons in the VTA. These studies raise important questions necessary to fully understand the interactions of the neuroendocrine and mesolimbic dopamine systems developmentally in response to maternal LG, and in adulthood in the production of maternal behaviors. Nonetheless, these findings provide important insights into the environmental and genetic contributions to the development of maternal behaviors.

Figure 5.5

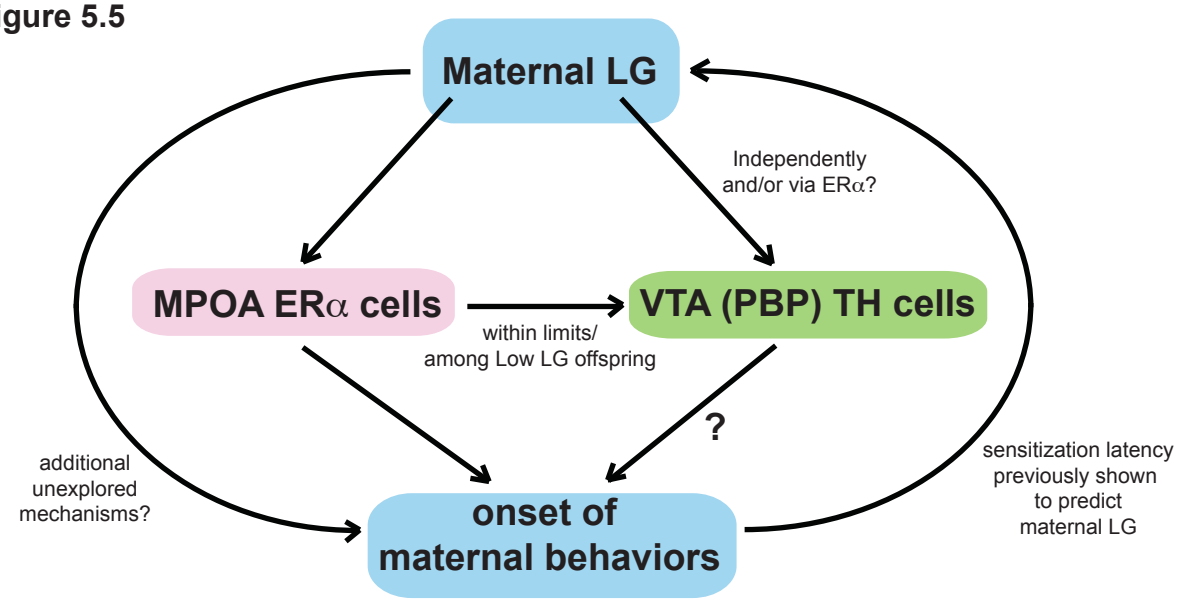

Figure 5.5 Model for the effects of maternal LG and ERα on offspring brain and behavior

Chapter 6: Summary and Discussion

Data summary and significance

The work presented in this thesis demonstrates that maternal care programs variation in offspring neuroendocrine and mesolimbic dopamine pathways during the postnatal period, and that these alterations are important for maternal and reward-directed behaviors. These findings contribute to our knowledge regarding the developmental impact of maternal behavior by 1) demonstrating the developmental time-course and epigenetic regulation of changes in the brain of female offspring associated with postnatal LG, 2) illustrating the behavioral consequences of these developmental changes, 3) revealing sensitive periods for maternal care effects on offspring gene expression and behavior, and 4) providing evidence that variation in ERα can have a direct impact on neurobiological and behavioral outcomes associated with postnatal maternal LG.

Maternal care as a moderator of offspring development

The results presented in this thesis clearly demonstrate that maternal care moderates several aspects of offspring brain development relevant to adult maternal behavior and other complex behaviors. Development of offspring maternal behavior may be particularly sensitive to variation in maternal LG in the early postnatal period, due to enhanced sensitivity of the MPOA to environmental stimuli during this time, as suggested by the ability of estradiol to

induce sexually-dimorphic changes in the MPOA until PN5 (Rhees et al., 1990b; DonCarlos and Handa, 1994; Al-Bader et al., 2008), and continued maturation of the mesolimbic dopamine system through the third postnatal week, also suggestive of enhanced sensitivity to environmental experiences during this time. Our finding of maternally-induced alterations emerging by PN6 in both the MPOA ERα and mesolimbic dopamine systems have implications not only for maternal behavior, but for other neuroendocrine and dopamine-dependent behaviors as well, which is also evident from our findings on maternally-induced alterations of reward-directed behavior. These findings are consistent with previous studies of the wide-ranging effects of maternal LG and of other paradigms examining the effects of disruptions to mother-infant interactions, particularly on stress sensitivity. For example, among male rats, maternal separation was previously found to increase dopamine responses to stress and enhance locomotor activity in response to cocaine (Meaney et al., 2002). Male offspring reared by High relative to Low LG dams were previously shown to have modest HPA response to acute stress, increased exploration in novel environments, and enhanced performance in tests of spatial learning and object recognition (Liu et al., 1997; Liu et al., 2000b; Bredy et al., 2003; Bredy et al., 2004; Weaver et al., 2004; Weaver, 2005; Toki et al., 2007). These stress and cognitive effects have been shown to be mediated by the hippocampus, and there is evidence that High LG male offspring have greater hippocampal glucocorticoid receptor expression and hippocampal synaptic plasticity (in the form of long-term potentiation, LTP) compared to Low LG offspring (Bredy et al., 2003; Broadbent et al., 2004; Champagne et al., 2008; Bagot et al., 2009).

Similarly, among humans early childhood adversity and low parent-child attachment are significant predictors of stress sensitivity, mood and psychiatric disorders, drug abuse, and poor physical health (Holmes and Robins, 1987; Riggs et al., 1990; De Bellis et al., 1994; Heim et al.,

1997; Russek and Schwartz, 1997; Bensley et al., 1999; Heim and Nemeroff, 2001; Heim et al., 2008; Hahm et al., 2010; Heim et al., 2010; Enoch, 2011; Scott et al., 2012). Some of these effects may likewise be mediated by hippocampal GR, as men who had experienced childhood adversity or abuse had lower hippocampal GR expression (McGowan et al., 2009). Identifying the timing of onset of neurobiological impairments in response to early life adversity has important implications for intervention, potentially allowing for targeted intervention at the time when these changes are occurring.

The current finding of a limited period of sensitivity to the maternal environment on offspring ERα gene expression and maternal behavior (Chapter 3) is relevant to the human practice of foster-care and adoptions. Children who are adopted after experiencing abuse or neglect generally score more favorably on behavioral, psychological, and cognitive measures compared to children who remained in abusive homes (Johnson, 2002; Macmillan et al., 2009). However, children with any history of maltreatment are more likely to have long-term behavioral and psychological difficulties compared to non-abused children, with increased risk associated with older age at adoption (Rutter, 1998; Rushton and Dance, 2006). Neuroanatomical alterations such as amygdala volume have also been associated with the duration of time spent in a substantially impoverished environment (Mehta et al., 2009). These studies indicate that the negative effects of maltreatment can be at least partially "rescued" by transitioning into a more positive home environment. Although it is clear that environmental experiences can affect the brain and behavior across the lifespan, clinical studies have also argued for sensitive periods to the quality of parental care in children. There is substantial evidence demonstrating maternal depression during a wide range of child's ages predicts negative child outcomes, but one recent study found that risk for child behavioral problems was predicted by having a mother with major

depression during the first year of a child's life, to a greater degree than having major depression prior to or during pregnancy (Bagner et al., 2010). Those findings were consistent with the rapid growth of emotion regulation and cognitive systems in the first year after birth (Bell and Wolfe, 2004), which was termed the sensitive period to emotional development (Bagner et al., 2010). Another recent study found that harsh and unpredictable home environments experienced from ages 0-5, but not ages 6-16, predicted sexual, aggressive, and criminal behavior by age 23 (Simpson et al., 2012). Together, these findings indicate greater sensitivity to the parental environment earlier in life, with later experiences only partially able to overcome neurobiological and behavioral effects of earlier life experiences.

Developmental interactions of hypothalamic ERα and the mesolimbic dopamine systems

Anatomical connections between the MPOA and the VTA have been previously demonstrated (Morrell et al., 1984; Shahrokh et al., 2010), and here we provide novel evidence for the developmental influence of MPOA ERα-ir cells on dopaminergic cells of the VTA, particularly within the PBP nucleus. Neurons projecting from the MPOA to the VTA have been shown to be estrogen-sensitive and oxytocinergic (Morrell et al., 1984; Shahrokh et al., 2010); **Figure 1.3**), and the direct effects of oxytocin on the VTA have been previously demonstrated (Pedersen et al., 1994; Febo, 2005; Shahrokh et al., 2010). These previous findings support an indirect role for ERα cells of the MPOA to affect dopaminergic neurons of the VTA, but controversy remained as to a role for ERα signaling on dopaminergic neurons given the lack of ERα in the adult VTA (Shughrue et al., 1997). Our finding of a correlation between the level of MPOA ERα-ir and PBP TH-ir cells among Low LG females, and a trend for elevated TH-ir cells

among Low LG offspring over-expressing ESR1, suggests a direct influence of ERα during early postnatal development, consistent with previous findings of transient ERα, ERß, and aromatase expression in the VTA until PN10 (Raab et al., 1995; Kritzer, 1997; Simerly et al., 1997; Karolczak et al., 1998; Christian and Gillies, 1999; Raab et al., 1999; Kuppers et al., 2001).

This potential developmental influence of ERα in the MPOA on the mesolimbic dopamine system has important long-term implications for other dopamine-mediated behaviors that may be hormonally influenced. Estrogen receptors and estrous cycle state have been shown to interact with D2 dopamine receptors to mediate social learning in female mice (Choleris et al., 2011), and oxytocin and D2 receptors were demonstrated to cooperate in pair bonding formation among female prairie voles (Liu and Wang, 2003). Interactions of ovarian hormones and the neuroendocrine system with the mesolimbic system have also been suggested to confer vulnerability of adolescent girls and women to drugs of abuse (Hedges et al., 2010). Women in the late follicular stage of the estrous cycle reported more "liking" of amphetamine, and women supplemented with estradiol in the early follicular phase reported more pleasant responses and more desire for amphetamine (Hedges et al., 2010). These findings suggest that the early maternal environment may predispose offspring to altered social and addictive behaviors through long-term alterations in neuroendocrine and mesolimbic dopamine pathways.

Estrogen/oxytocin-dopamine interactions have also been found to regulate sexual receptivity and behavior in both male and female rodents (Melis et al., 2007; Baskerville and Douglas, 2008; Graham and Pfaus, 2010; Graham and Pfaus, 2012). Among males, penile erection was elicited with oxytocin injection into the VTA, and blocked by injection of an OTR antagonist in the VTA or injection of haloperidol into the NAc shell (Melis et al., 2007). Among females, hormonal priming with estrogen is necessary for sexual receptivity, which is also

dependent upon dopamine signaling in the NAc (Graham and Pfaus, 2010; Graham and Pfaus, 2012). Variation in female sexual receptivity has in fact been demonstrated among offspring of High and Low LG dams. When females were able to control (pace) mating, Low compared to High LG female offspring showed increased sexual receptivity, including higher lordosis rating, shorter inter-intromission interval, received more ejaculations, and had a greater probability of pregnancy (Cameron et al., 2008a; Cameron et al., 2008b). These maternal LG group differences in behavior were accompanied by neuroendocrine differences associated with sexual receptivity, including increased levels of lutenizing hormone and progesterone at proestrus, an increased effect of estradiol on gonadotropin releasing-hormone in the MPOA, and increased ERα-ir in the anterioventral PVN and ventrolateral VMH among Low compared to High LG offspring (Rosenblatt et al., 1994; Cameron et al., 2008a; Cameron et al., 2011). While not directly tested by these studies, our findings implicate a potential role for maternally-induced variation in the mesolimbic dopamine system for female sexual behavior, and it would be interesting to test the conditioned place preference, and dopamine receptor dependence, of Low and High LG females for sexual behavior.

Furthermore, there is evidence for parental effects on human female reproductive development. An impoverished home environment was found to predict earlier onset of puberty and sexual activity, increased numbers of sexual partners in adolescence, and younger age at first pregnancy, while increased positive parent-child interaction time was associated with later onset of sexual maturation (Phinney et al., 1990; Belsky et al., 1991; Graber et al., 1995; Kim and Smith, 1998; Ellis et al., 1999; Ellis, 2004; Nettle et al., 2011; Simpson et al., 2012). Pregnancy in adolescence is further associated with stress, impaired health, low social support, impoverished life style, and higher risk for child maltreatment (Davis, 1989; Patch, 1990),

perpetuating these effects to the next generation. These and the results indicated in this thesis suggest that hormone and dopamine-regulated behaviors such as sexual and maternal behavior emerge during postnatal development prior to puberty, indicating that early interventions may be most effective in altering such behaviors.

Epigenetic effects of early environmental experiences

The results of these studies demonstrate that maternal LG influences postnatal ERα levels by two distinct epigenetic mechanisms, although *Th* was not differentially methylated in response to maternal LG. LG group differences in *Esr1* B/1b CpG methylation found here are consistent with previous reports of decreased levels of CpG methylation among adult High LG offspring (Champagne et al., 2006). However, LG-mediated epigenetic regulation of *Esr1* by chromatin remodeling had not previously been explored, and to our knowledge this is the first study to examine the epigenetic regulation of *Esr1* by chromatin remodeling at multiple postnatal stages. There are now many examples of environmental modulation of epigenetic gene regulation among both humans and animals (reviewed in Chapter 1), although relatively few studies that have explored the developmental time course of these epigenetic effects.

The importance of examining epigenetic regulation of gene expression across postnatal development has been demonstrated by studies showing increases and subsequent decreases in methylation in response to environmental experience. For example, DNA methylation was found to be reversible among honeybees when changing between forager and nurse castes (Herb et al., 2012), which may be dependent upon dietary methyl donors (Ford 2012 Exp Gerontol). Another study examined hippocampal GR 1_7 CpG methylation levels among male offspring of Low and High LG dams, and found methylation at one CpG site in the promoter was

significantly elevated between E20 and PN1 among both Low and High LG offspring, but was subsequently reduced again at PN7 and through adulthood only among Low LG offspring (Weaver et al., 2004). This finding was controversial and challenged the idea that methylation is stable across the lifespan, and would not have been detected if CpG methylation was only examined in adulthood. Furthermore, in studies attempting to elucidate the impact of the maternal environment on epigenetic regulation of gene expression, it is necessary to examine levels of gene expression and epigenetic marks at birth in order to confirm that any alterations are due to the postnatal environment rather than *in utero* effects.

While the gene silencing role of DNA methylation has been demonstrated (Fan et al., 2006; Giacinti et al., 2006; Wei et al., 2008), it is important to consider various means of gene regulation. This is particularly true in complicated *in vivo* systems, and because it is unknown whether total promoter methylation, or methylation at specific CpG sites, has the greatest repressive effect on gene expression. For example, arginine vasopressing (AVP) gene expression was found to be correlated with the level of methylation at some but not all CpG sites in AVP's enhancer region, and the degree of correlation was reduced in response to early life stress (Murgatroyd et al., 2009), and other studies have found alterations in gene expression to be better explained by chromatin modifications than promoter methylation (He et al., 2011). DNA methylation has further been shown to interact with histone modifications, such that DNMTs and methyl CpG binding proteins (such as MeCP2) affect chromatin by interaction with HDACs, and H3K9 methylation has been shown to interact with DNMTs to promote CpG methylation and repress gene expression (Fuks, 2005). Therefore, the absence of LG group differences in *Th* promoter methylation across development suggests that we would likewise not find maternally-

induced variation in chromatin remodeling around *Th*, although this would be need to be confirmed in future chromatin immunoprecipitation studies.

Parental transmission of effects across generations

The results from these studies enhance our understanding of the mechanisms by which experience of maternal care is translated into the later expression of maternal care. We directly demonstrate that increased levels of ERα in the MPOA of Low LG offspring facilitates onset of maternal behavior, suggesting that ERα *mediates* the effect of maternal LG on offspring maternal behavior, although whether VTA dopaminergic cells likewise mediate the effect of maternal LG on offspring has yet to be clearly determined (**Figure 6.1**). Thus, the level of maternal LG is perpetuated from one generation to the next. This transmission of behavior is thought to be germline-independent, as cross-fostering at birth (Francis et al., 1999) or at PN6 (Chapter 3) reverses the effect of the biological mother on offspring maternal behavior. Transmission of parenting style across generations has also been demonstrated in humans and non-human primates. Rhesus macaques reared without a mother and postnatally isolated displayed abusive and neglectful maternal behaviors towards offspring, which could be at least partially reversed by later social exposure or extended contact with infants (Seay et al., 1964; Harlow, 1965; Arling and Harlow, 1967; Harlow and Suomi, 1971). Abusive or protective parenting among macaques was found to be consistent across generations, and matched that of a foster parent, indicating experience as a mediating factor (Maestripieri et al., 1999; Maestripieri, 2003; Maestripieri et al., 2007). Likewise, in humans, experience of neglect, maltreatment, physical abuse, and sexual abuse during childhood are predictive of maltreatment behaviors towards their own children (Newcomb and Locke, 2001; Bifulco et al., 2002; Lev-Wiesel, 2006; Berlin et al., 2011). Within

the normal range of parenting behavior, infant attachment is likewise predicted by the attachment scores, sensitivity, and intrusiveness of mothers and grandmothers (Benoit and Parker, 1994; Kretchmar and Jacobvitz, 2002). The results of the studies within this thesis suggest that epigenetic and transcriptional mechanisms, within both hormonal and motivational neural systems, mediate these transgenerational effects of maternal behavior.

Figure 6.1

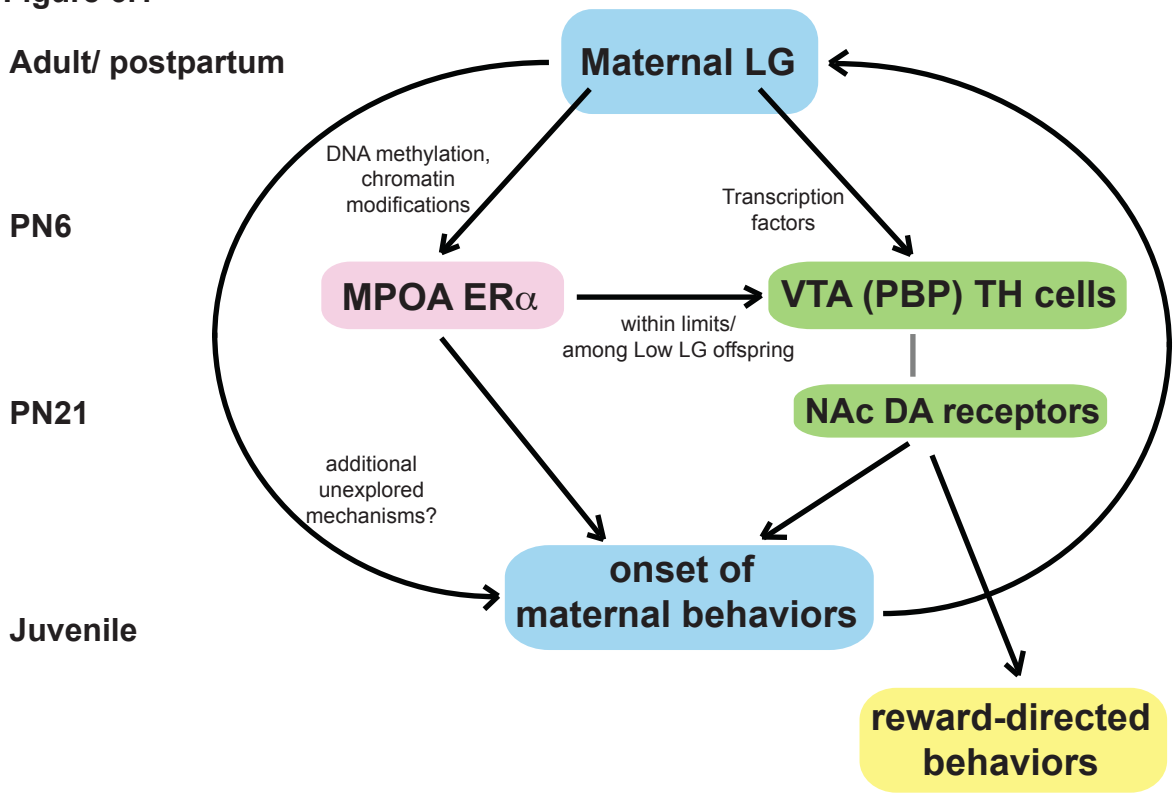

Figure 6.1 An updated model of mechanisms mediating transmission of maternal behavior from mothers to daughters, via effects on MPOA ERα and VTA dopamine neurons

Future directions

These findings raise intriguing questions to be addressed in future studies. In particular more research is needed to determine 1) the causal effects of DNA methylation and post-translational histone modifications on offspring maternal behavior, and 2) the role of variation in the mesolimbic dopamine system on MPOA ERα and maternal behavior.

Maternally-induced variation in *Esr1* B/1b methylation and histone modifications was associated with the levels of ERα mRNA, but the dependence of offspring maternal behavior on epigenetic modifications established in the postnatal period is unknown. This could be assessed in future studies by administering pharmacological agents known to disrupt the epigenetic state, such as the histone deacetylase inhibitor trichostatin-A (TSA) or the DNA methylation inhibitor 5-azacytadine (5-aza). Although the acetylation state of *Esr1*-associated chromatin was not tested, both TSA and 5-aza have been shown to decrease levels of DNA and H3K9 methylation (Yang et al., 2010). Maternal sensitization testing in Low and High LG offspring, before and after treatment with either of these compounds, would give insight into whether maternally-induced epigenetic regulation of *Esr1* mediates offspring maternal behavior. Caveats of such a study include the broad effects of the HDAC inhibitors, even if delivered locally to the MPOA. However, ICV infusion of TSA over 7 days has previously been demonstrated to elevate GR methylation and protein levels, as well as open field behavior, among Low LG male offspring without severely disrupting all systems (Weaver et al., 2004), suggesting that TSA could be used successfully until more selective means for epigenetically manipulating specific target genes are developed.

Finally, the developmental interactions of ERα in the MPOA, dopamine neurons in the VTA, and maternal behavior should be further examined. We showed that developmentally

increasing *Esr1* in the MPOA led to elevated TH-ir cells in the VTA and decreased latency to onset of maternal behavior among Low LG offspring. However, it remains to be determined whether developmentally enhancing levels of dopamine neurons in the VTA would similarly impact levels of ERα-ir cells in the MPOA and/or maternal behavior. In other words, is the developmental interaction of the hypothalamic neuroendocrine and mesolimbic dopamine systems bi-directional? While it would be informative if increased levels of dopamine neurons in the VTA led to enhanced maternal behavior, the participation of the mesolimbic dopamine system in a diversity of functions and neuropsychiatric and degenerative disorders would necessitate comprehensive behavioral testing for a variety of motivated, attention-dependent, and cognitive behaviors. Additionally, the timing of the effects of *Esr1* over-expression on midbrain dopamine development should be further examined. Temporary over-expression of *Esr1* in the MPOA, only during the pre-weaning period, would elucidate whether the anatomical and behavioral alterations found subsequent to prolonged over-expression were organizational (permanent changes to the system induced within a discrete period of time that) or whether they require ongoing elevations to the level of ERα. *Esr1* over-expression beginning in adulthood would answer the question of whether elevated levels of ERα in the MPOA at *any* time during development are sufficient to drive changes in VTA dopamine neurons and maternal behavior. Nevertheless, the results of the studies within this thesis have significantly enhanced our understanding of the mechanisms and timing by which maternal care programs variation in offspring neuroendocrine and mesolimbic dopamine pathways during the postnatal period.

Appendix 1: List of abbreviations

Brain regions

Arc	arcuate nucleus of the hypothalamus
BNST	bed nucleus of the stria terminalis
Cg2	cingulate cortex
CLi	caudal linear nucleus of the raphe
IF	interfascicular nucleus
MeA	medial amygdala
mPFC	medial prefrontal cortex
MPOA	medial preoptic area
NAc	nucleus accumbens
PBP	parabrachial pigmented nucleus of the VTA
Pe	periventricular nucleus of the hypothalamus
PIF	parainterfascicular nucleus of the VTA
Pir	piriform cortex
PN	paranigral nucleus of the VTA
PVN, Pa	paraventricular nucleus of the hypothalamus
RLi	rostral linear nucleus of the raphe
S1	primary somatosensory cortex
SNCD	substantia nigra pars compacta, dorsal tier
SNCM	substantia nigra pars compacta, medial tier
SNCV	substantia nigra pars compacta, ventral tier
SNL	substantia nigra pars lateralis
SNR	substantia nigra pars reticulata
TuL	lateral tubercle
VMH	ventral medial hypothalamus
VTA	ventral tegmental area
VTAR	rostral VTA; parafasciculus retroflexus area by Ikemoto (2007)

Proteins/ genes, and molecules

Ad-	adenovirus
AVP	arginine vasopressin
BDNF	brain derived neurotrophic factor
Cdkn1c / p57kip2	cyclin-dependent kinase inhibitor n1c
CRF	corticotrophin-releasing-factor
D1 / Drd1	dopamine receptor type 1
D2 / Drd2	dopamine receptor type 2
D3 / Drd3	dopamine receptor type 3
DA	dopamine
DAT	dopamine transporter
DNMT1	DNA methyltransferase 1
DNMT3a	DNA methyltransferase 3a
E2	estradiol
ERE	estrogen response element
ERß	estrogen receptor-beta
ERα / Esr1	estrogen receptor-alpha
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
H3K4	histone 3, lysine 4
H3K9	histone 3, lysine 9
HAT	histone acetyl transferase
HDAC	histone deacetylase
HMT	histone methyltransferase
Lmx1b	LIM homeodomain family member 1b
me3	tri-methylation
Nurr1 / Nr4a2	Nuclear receptor related 1 protein
OT	oxytocin
OTR	oxytocin receptor
PIK3	Phosphatidylinositol 3-kinase

Pitx3	paired-like homeodomain transcription factor
PKA, PKC	protein kinase type A or C
PLC	phospholipase C
Stat5b	signal transducer and activator of transcription
TH	tyrosine hydroxylase
TrkB	neurotrophic tyrosine kinase receptor, type 2

Behaviors, tests, and other

LG	licking/ grooming
CpG	cytosine-guanine pair
CPP	conditioned place preference
E (#)	embryonic day
PN (#)	postnatal day
fMRI BOLD	functional magnetic resonance imaging blood oxygen level dependent contrast

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